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der Vetsuisse-Fakultät Universität Zürich

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Tandem purification of Mre11/Rad50-dimers using the Baculovirus Expression System

Inaugural-Dissertation

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Index of contents

1. Summary

The Mre11/Rad50/Nbs1-complex (MRN) is activated in response to DNA double-strand breaks (DSB). DSBs belong to the most toxic forms of DNA-damage, they lead to the activation of the DNA damage response (DDR), which ultimately results in the activation of cell cycle checkpoints, coordination of DNA repair mechanisms, induction of transcriptional programs or apoptosis and senescence if the damage is too severe. The two main DNA repair pathways to overcome DSBs are non-homologues end joining (NHEJ) and homologues recombination (HR). MRN is involved as sensor of DSBs, it binds to DSBs and is recruited to DSB-flanking chromatin compartments. Our lab has purified MRN and MR subcomplexes from Baculovirus-infected insect cells and our collaborator analysed these by single-particle electron microscopy (EM). The analysis revealed that the MR heterodimers were present as monomers, multimers and protein aggregates and it was not possible to separate and properly analyse them. Thus, the goal of this project was to isolate and purify MR heterodimers for subsequent EM analysis. For this, differentially-tagged Mre11 and Rad50 were co-expressed in insect cells and tandem purified. While the original goal was not achieved due to technical difficulties and time constraints, the result of this thesis revealed that polyhistidine-tagged Rad50 co-purifies with untagged Mre11, which indicates that these two human proteins form a complex when co-expressed in insect cells.

Keywords: MRN, tandem purification, Baculovirus Expression System, DNA-Repair

2. Introduction

3 billion base pairs of DNA form the human genome, a sensitive entity complex with various proteins and located in the cell nucleus. Even small alterations can cause serious consequences; therefore, the maintenance of the genome is of great importance for a cell. Through a single mutation, severe developmental disorders or cancer formation can occur. The cells in all organisms are constantly under influence of endogenous and exogenous agents like ultraviolet light, ionizing radiation and genotoxic agents that can induce DNA damage; if the induced damage is not repaired properly, mutations can occur (Hoeijmakers, 2001).

If critical genes are mutated, cancer formation can be promoted (Vogelstein and Kinzler, 2004). In order to protect the genetic information from the deleterious effects of DNA damage, various mechanisms evolved in the cells to react in an appropriated manner. Many proteins are involved in the DNA damage response (DDR); they detect, signal the presence of, and repair DNA damage. They also coordinate the cell cycle progression to ensure correct DNA replication and cell division. The response can lead to the activation of cell cycle checkpoints, DNA repair mechanisms, transcriptional programs, apoptosis and senescence (Rupnik et al., 2010). On the level of the whole organism, unrepaired DNA damage can lead to immunodeficiency, premature aging and neurodegeneration (Ljungman, 2010).
Various forms of DNA damage can occur; one of the most toxic forms is DNA double-strand breaks (DSBs) because both strands of the DNA are affected and thus, there is no intact strand left that can serve as a template for the repair synthesis. Several agents can lead to double-strand breaks, most prominent are ionizing radiation (IR) or radiomimetic chemicals, but also mechanic stress on chromosomes, products of collapsed replication forks, or even the physiological event of V(D)J recombination in B- and T-lymphocyte development can lead to DSB formation. The repair is so difficult because the structure of the DNA double helix is broken and even the short complementary overhangs cannot keep the DNA ends juxtaposed by base pairing and the ends may dissociate from each other if not held together by means of DNA end-binding protein complexes. It has been shown that DSBs can lead to induction of mutations and chromosomal translocation, which are hallmarks of tumor cells (Jackson, 2002).

2.1. DNA double-strand breaks

2.1.1. DSB repair mechanisms

DSB repair mechanisms are highly conserved throughout evolution, they are found in phages, bacteria, yeast and higher eukaryotes (Cromie et al., 2001). Homologues recombination (HR) and non-homologues end joining (NHEJ) are two main repair pathways for DSBs. In mammalian cells, the HR predominates mainly in S- and G2 phases of the cell cycle when the undamaged sister chromatid can be used as a template, whereas NHEJ predominates in G0- and G1-phase (Shrivastav et al., 2008).

2.1.2. Homologues recombination

For the HR, homologous sequences in form of sister chromatids, homologous chromosomes or DNA repeats are required. It is considered an error-free repair pathway (Takata et al., 1998). First, the DNA ends are resected to yield 3' single-stranded overhangs. This resection is initiated by the MRN complex and regulated by CtIP, an adaptor protein. The resection is then completed by the nuclease EXO1 and several helicases (Paull and Gellert, 1998). After resection, Rad52 and Rad54 promote ATP-dependent homologous DNA pairing and strand exchange (Baumann et al., 1996; Gupta et al., 1997). RPA coats the ssDNA tails, Rad51 forms a complex with it and initiates strand exchange (Sung, 1997). BRCA2 interacts with Rad51 and RPA at DSBs and helps to load Rad51 onto the DNA (Yang et al., 2002). BRCA1 and BRCA2 are believed to be important at early steps of HR, perhaps because they coordinate DNA repair with other cellular processes. Following sister-chromatid pairing and strand invasion, two distinct pathways can be used. Favoured in mammalian cells is the non-crossing-over, it results from disengagement of the Holliday junction followed by DNA pairing and gap filling in the damaged homologue (Johnson and Jasin, 2000). In the classical path the Holliday junctions are resolved by endonucleolytic cleavage and the probability of crossover or noncrossover are equal. Many factors that are involved in HR are essential. HR may be more important in embryonic cells than in normal adult somatic cells, and studies suggest that disruption of factors involved in HR result in chromosomal instability and cancer predisposition (Thompson and Schild, 2001; van Gent et al., 2001). (Figure 1B)

2.1.3. Non-homologues end joining

NHEJ is the predominant repair mechanism in higher eukaryotes, but is not as accurate as HR. By adding or deleting nucleotides from free DNA ends, the strands are processed till the ends can be joined. For this process, no sister chromatid is needed (Budman and Chu, 2005). The Ku heterodimer that consists of Ku70 and Ku80 binds free DNA ends and recruits the DNA-PKcs, which then becomes activated (Martensson and Hammarsten, 2002). Analyses of the catalytic domains revealed DNA-PKcs to be a member of the phosphatidylinositol 3-kinase-like family (PIKK) that also includes ATM (ataxia telangiectasia mutated) and ATR (ATM related) (Hartley et al., 1995). Further studies showed different interactions of DNA-PKcs with other proteins like Artemis, which is activated through phosphorylation that induces an endonuclease hairpin opening activity (Ma et al., 2002; Riha et al., 2006). Loss or mutation of DNA-PKcs leads to radio hypersensitivity and severe combined immunodeficiency.

After recruitment of DNA-PK and DNA end processing by Artemis and perhaps MRN, the Lig4/XRCC4 along with XLF joins the two DNA ends and restores the DNA structure (Ma et al., 2002). (Figure 1A)

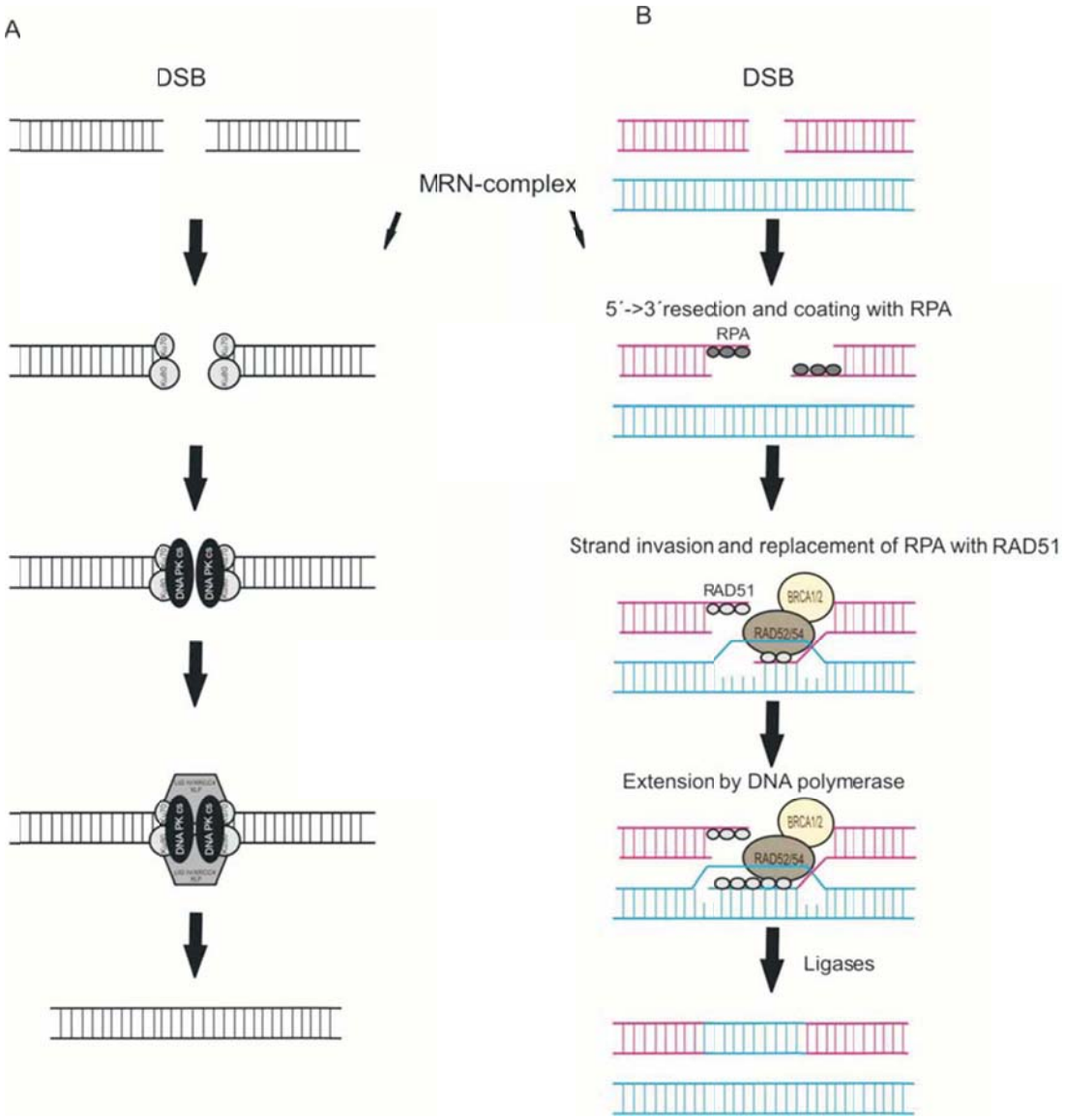

Figure 1: NHEJ (A) and HR (B) in DSB repair.

(Adapted from Czornak et al., J Appl Genet, 2008)

A: In NHEJ the DSB is recognized by KU70/80 which forms a complex with DNA PKcs. After recruitment of DNA-PK and processing by Artemis and perhaps MRN, the Lig4/XRCC4 along with XLF joins the two DNA ends and restores the DNA structure. B: In HR the DNA ends are resected by MRN and regulated by CtIP (not shown in figure), an adaptor protein and then completed by nuclease EXO1 and several helicases. RPA is loaded onto the DNA and then exchanged with the help of Rad52/54 and BRCA1/2 to Rad51. The ssDNA strand is then able to invade the sisterchromatid and DNA polymerases can fill up the gaps. The holiday junctions are resolved by endonuclolytic cleavage.

2.2. Cellular response to DNA double-strand breaks

In addition to the activation of the repair pathways, also a global cellular response that involves the entire cell is activated by DSBs. This includes the regulation of cell cycle transitions, transcriptional activation and apoptosis. First, sensor proteins detect the DSB and activate the kinase-dependent signalling cascade. The DNA damage response (DDR) is a signal-transduction pathway with sensors, transducers and effectors. The signal is amplified and diversified so that one single DSB can lead to a full cell cycle arrest (Khanna and Jackson, 2001).

ATM is a conserved signalling component with a central role in the cellular response to DSBs. Patients carrying two mutated ATM alleles manifest progressive cerebellar ataxia, immune deficiencies, gonadal atrophy, oculocutaneous telangiectasias, radiation sensitivity, premature ageing and increased risk of cancer (Bakkenist and Kastan, 2003). ATM belongs like DNA-PKcs to the PIKK family; it gets recruited to DNA DSB by the MRN complex, consisting of Mre11, Rad50 and Nbs1 (Stucki and Jackson, 2004; Falck et al., 2005). MRN accumulates rapidly at the damaged site, its activation leads to ATM autophosphorylation on multiple residues. The thus generated ATM monomers phosphorylate different targets such as checkpoint kinase 2 (CHK2), mediator of DNA damage checkpoint 1 (MDC1), p53 binding protein 1 (53bp1), breast cancer susceptibility protein (BRCA1) and histone 2A variant X (H2AX). Not only the repair pathways can be activated and regulated by these phosphorylations, it can also lead to transient or permanent cell cycle arrest if the damage persists, or to programmed cell death (apoptosis) when the DNA damage is too severe. The phosphorylation of H2AX leads to the recruitment of MDC1 to the site of DNA damage. MDC1 amplifies H2AX phosphorylation, maybe by recruiting ATM into the DSB-flanking chromatin compartment or by preventing dephosphorylation (Stucki and Jackson, 2006). For the interaction between MRN and MDC1, the FHA and the two BRCT domains of Nbs1 are needed, mutations in either of them leads to defects in MRN accumulation (Hari et al., 2010). Many proteins are recruited to the modified chromatin regions flanking DSBs. These regions can be visualized by standard fluorescence microscopy as bright speckles, usually termed DNA damage foci or ionizing radiation-induced foci (IRIF). The DNA damage foci are composed of many different factors that are mainly recruited through MDC1 and phosphorylated H2AX. It was shown that MDC1 recruits Ubc13-Rnf8, an ubiquitin ligase complex that ubiquitinylates different chromatin components at DSBs, and their poly-ubiquitination is needed for the recruitment of 53bp1 and BRCA1 (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). ATM or CHK1 and CHK2 target the effector proteins such as p53 and Cdc25a. These are major effector proteins that are often mutated in cancer cells. p53 acts as a transcription factor regulating expression of p21, a Cdk inhibitor protein responsible for G1 cell cycle ar-

rest, and its accumulation can lead to apoptosis through activation of the caspase-signaling cascade. Cdc25a acts as a phosphatase, removing the inhibitory phosphorylation on cyclin-dependent kinases (Cdks). Upon targeting of Cdc25a by CHK1 and CHK2 it is degraded and thus, Cdks such as the S-phase driving Cdk2 remain inactive.

In summary, DSBs can trigger different cellular responses dependent on the pathways that are activated. As mentioned above, one key player in the response to DSBs is the MRN complex, which was investigated in this thesis.

2.3. The MRE11/RAD50/NBS1 complex

The MRN complex has many tasks in the cellular response to DSBs. It acts as a sensor of DSBs and activator of signal transduction, it can influence the DSB repair pathway selection to HR or NHEJ, it has a repair function in HR and NHEJ and it takes part in the telomere homeostasis (Lamarche et al., 2010). (Figure 2)

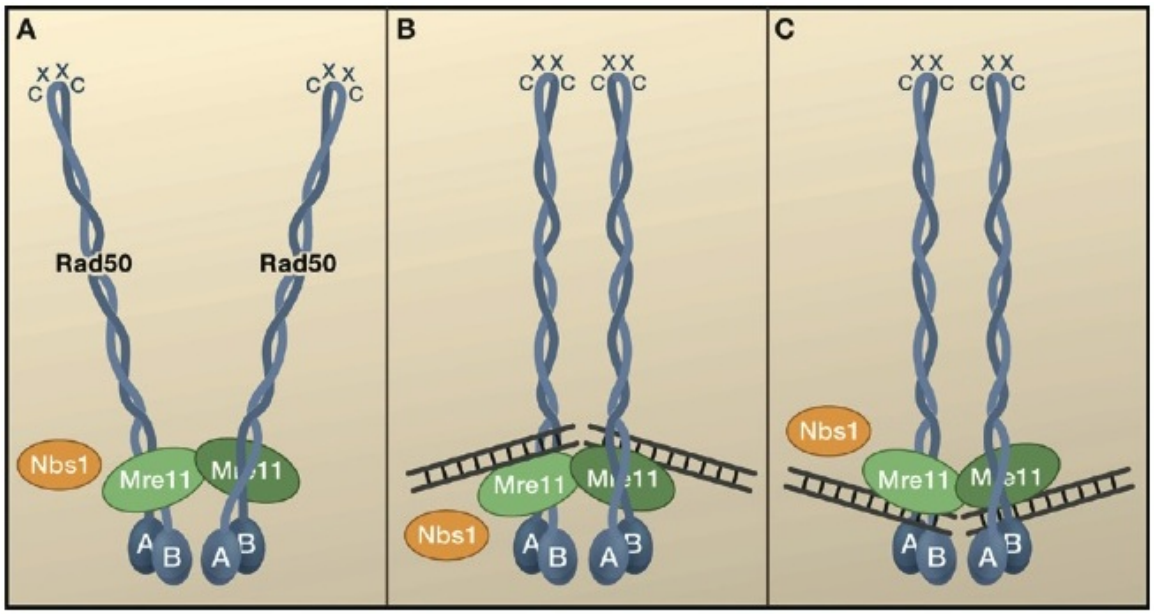

Figure 2: MRN Complex (A) and binding to the DNA (B/C)

(Reproduced from Kanaar. Cell, 2008)

The MRN complex is normally present as M2R2N1. The Walker A/B domains of Rad50 and the Mre11 build the globular domain. While the Rad50 coiled-coil region build long arms (A). Upon DNA binding it comes to conformational changes (B/C)

2.3.1. Mre11

The Mre11 protein forms a U-shaped dimer, which is required for DNA binding. It interacts with Rad50 via a C-terminal domain, flanked by two DNA binding regions. The Nbs1 interaction occurs via an N-terminally located interaction domain. The nuclease activity is located within conserved motifs in the N-terminal region (Rupnik et al., 2010). Mre11 can build up two structure type of interaction, first, the "synaptic DNA complex" mimicking DNA end joining with a 2bp 3' overhang on double-stranded oligonucleotides or second, the "branched DNA complex" that is formed between Mre11 and 'Y-shaped' oligonucleotides containing regions of both double and single-stranded DNA oligonucleotides. The binding is mediated entirely by minor groove

contacts to the sugar phosphate backbone, whereby Mre11 can work as a universal DNA end processing enzyme, since it doesn't need a specific sequence to bind (Williams et al., 2008; Furuse et al., 1998). But it prefers to bind to ds-oligonucleotides than to ss-oligonucleotides, and longer DNA substrates are also favoured. It prefers 3' overhangs to 5' overhangs and in ds-oligonucleotides, the binding is better with a cohesive overhang (Furuse et al., 1998; Usui et al., 1998). Mre11 shows no activity with duplex linear or circular substrates (Moreau et al., 1999).
For DNA binding the dimerization of Mre11 is important, while the endonuclease activity and the 3' to 5' exonuclease activity are not dependent on the high-affinity DNA binding through the dimerization (Williams et al., 2008). It was shown that the N-terminal phosphoesterase domain is not required for the nuclease activity (Furuse et al., 1998; Usui et al., 1998). The 3' to 5' exonuclease activity is dependent on specific DNA-alignment, for the 3' to 5' exonuclease activity the Motif II is needed, while the Motif III is required for endonuclease and exonuclease activity (Williams et al., 2008; Moreau et al., 1999; Usui et al., 1998). The exonuclease shows a weak 3' to 5' activity on a linear duplex substrate with Mn^{2+} as a cofactor (Moreau et al., 1999), but is independent of ATP (Furuse et al., 1998). For the early HR the endonuclease activity is crucial, it acts with additional factors together to open DNA ends and produce an effective 5' to 3' excision that liberates 3' ssDNA for RPA loading and initiation of strand invasion (Williams et al., 2008; Usui et al., 1998). The Mre11 endonuclease activity plays an essential role in meiosis, were it probably removes Spo11 from 5'ends of double-strand break sites. For vegetative growth, mating type switching, NHEJ and telomere maintenance, the nuclease activity is dispensable, because the DSBs can be processed by other nucleases, which act redundantly with Mre11 (Moreau et al., 1999). (Figure 3)

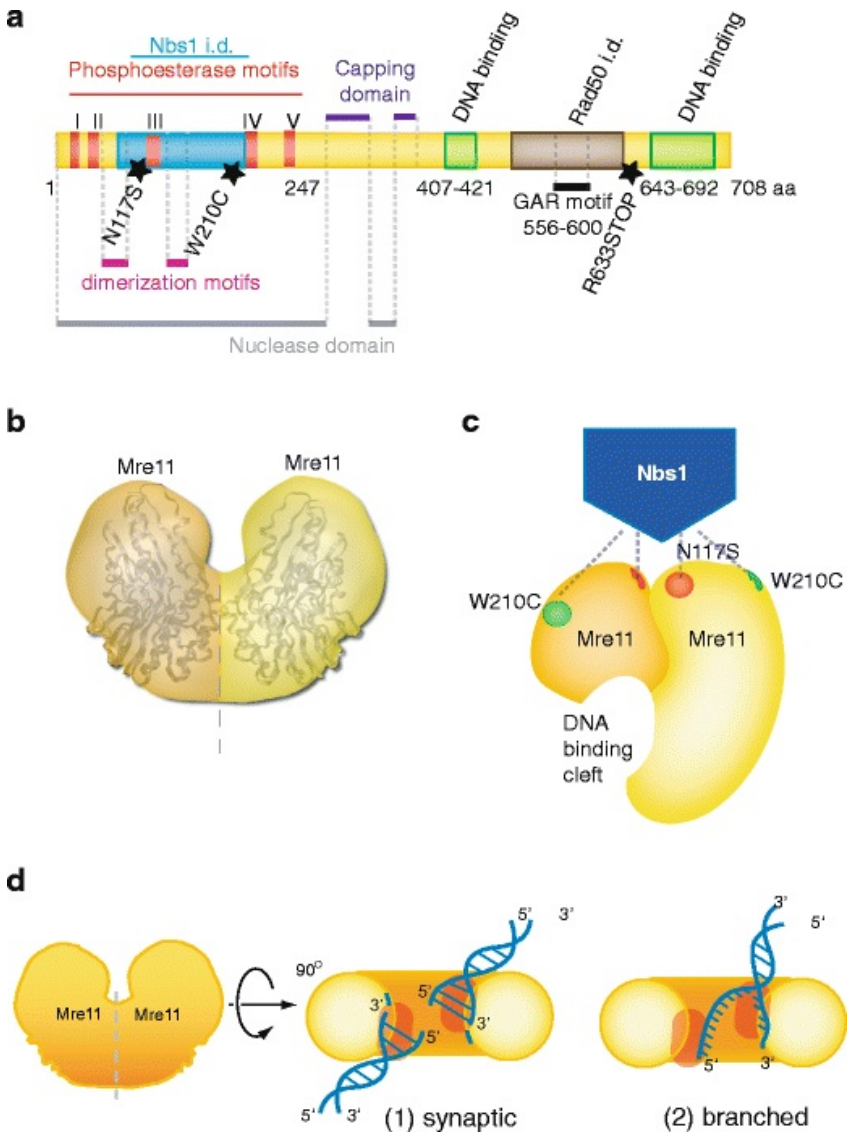

Figure 3: Structure of Mre11

(Reproduced from Rupnik, Chromosoma, 2010)

The domain structure of Mre11 (a), the „capping domain" is responsible for conformational changes upon DNA binding. Indicated with stars are mutations found in ataxia telangiectasia-like disorder (ATLD). Mre11 forms a U shaped dimer (b). Nbs1 interaction with Mre11 (c). Interaction types of Mre11 (d).

2.3.2. Rad50 (MR)

Rad50 is a member of the structural maintenance of chromosome (SMC) family, involved in chromosome condensation and sister-chromatid cohesion. Both termini contain Walker A and B motifs required for ATPase activity, DNA binding and partial DNA unwinding. Studies showed that mutations in the Walker A motif cause hypersensitivity to DNA damaging agents, which was as sensitive as Rad50 knockout. The mutants are compromised for HR and NHEJ. The

complex assembly is not defective in the mutant but the ATP hydrolysis is inaccurate. Thus, the walker motifs are not involved in complex formation (Chen et al., 2008).

The large coiled –coil structure in the central region can fold back and allows both Walker motifs to interact directly. This interaction forms a globular ABC transporter-type ATPase domain. The Mre11 subunit binds to the coiled-coil region near the ATPase domain and it was shown that the ATPase activity of Rad50 is required to form the complex with Mre11. Rad50 molecules can dimerize through the "hinge" region at the end of the coiled-coil arm. This arm contains a zinc hook with the Cys-X-X-Cys (CXXC) motif; this architectural structure suggests that two Rad50 form a metal-mediated bridging complex between two DNA-binding heads. The dimerization is shown in the presence of Zn^{2+}, the MR complex can build a lot of different complexes and shapes that are important for the different cellular functions of the complex. If the conserved cysteine is mutated it leads to a higher sensitivity to ionizing radiation (Hopfner et al., 2002). The length and conformation also suggest the role to link sister chromatids in homologous recombination and DNA ends in non-homologous end joining. But the dimerization of the Rad50 hook domain is not necessary for DNA binding (Cahill and Carney, 2007).

Like other ABC transporter superfamily members, Rad50 has the activity to reversible transfer a phosphate group from ATP to AMP to generate two ADPs. The ATPase and adenylate kinase activities act competitively and may be regulate conformational changes required for the activity of Rad50. The adenylate kinase activity is required for DNA tethering.

Rad50 can enhance the nuclease activity of Mre11, while ATP shows no effect and also the exonuclease activity of Mre11 is not affected (Chen et al., 2008). It was also shown that Rad50 itself has no nuclease activity, so that the increased nuclease activity in the MR complex does not come from the Rad50 protein itself. Mre11 binds next to the Walker A and Walker B motifs in the coiled-coil region of Rad50 (Hopfner et al., 2002; Paull and Gellert, 1998). The MR complex works in an ATP-dependent manner in DNA end processing in meiosis and mitotic DSB repair. Through conformational changes in Rad50, the DNA substrate is prepared for cleavage by Mre11, and afterwards the DNA is released upon ATP hydrolysis along with the reversal of the Rad50 conformation (Hopfner et al., 2002). (Figure 4)

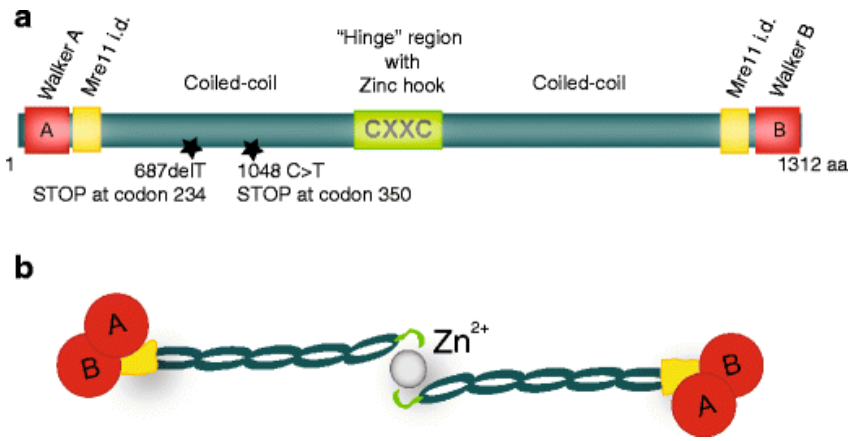

Figure 4: Structure of Rad50.

(Reproduced from Rupnik, Chromosoma, 2010)

Domain structure of Rad50. Indicated with stars are known mutation in Rad50 (a). Schematic dimerization of Rad50 (b).

2.3.3. Nbs1

Nbs1 is thought to regulate the MR functions, it is required for the nuclear localisation of Mre11 and Rad50, and it is also needed for the rapid focal assembly of the MRN complex to the sites of DNA damage. It stimulates the activities of Rad50 and Mre11 such as nuclease activity and ATP-dependent DNA unwinding (Rupnik et al., 2010).

The protein was found mutated in patients with the Nijmegen breakage syndrome (NBS), which is an autosomal recessive disorder that leads to sensitive to irradiation, chromosome fragility and fails to activate cell cycle checkpoints in response to DNA damage. In the patient's cells no MRN nuclear foci are formed following the induction of DSB by ionizing radiation, because in most NBS patients, only a truncated form of NBS1 (called p70) is expressed, that cannot bind to MDC1 (Carney et al., 1998).

Nbs1 has an important role in the modulation of ATM activation in the irradiation induced signalling cascade, through interaction with Mre11, it modulate the timing and the amplitude of ATM activation in response to low doses of IR (Horejsi et al., 2004). In NBS patients the phosphorylation of Chk2, Smc1 and Fancd2 by ATM is reduced or absent. Through multiple contacts with MRN a conformational change in ATM is induced, which increases its affinity for its substrates (Lee and Paull, 2004).

Nbs1 also modulates the DNA binding activity and specificity of Rad50. Nucleotide-dependent DNA binding is strongly promoted when Nbs1 is present, this increased DNA binding activity may explain the requirement for Nbs1 in DNA unwinding (Lee et al., 2003).

Depending on the stoichiometry of Mre11 and Nbs1, different MRN sub-complexes are formed. Rad50 can be present as monomer, dimer or multimer in complex with Mre11 and Nbs1. If Nbs1 is in abundance compared to Mre11, a complex containing Rad50 multimers is preferably formed while equal amounts seems to lead to the formation of Rad50 monomers (van der Linden et al., 2009). These different forms are one explanation why this complex has a lot of different functions and that the stoichiometry of MRN is important to consider in structural studies. (Figure 5)

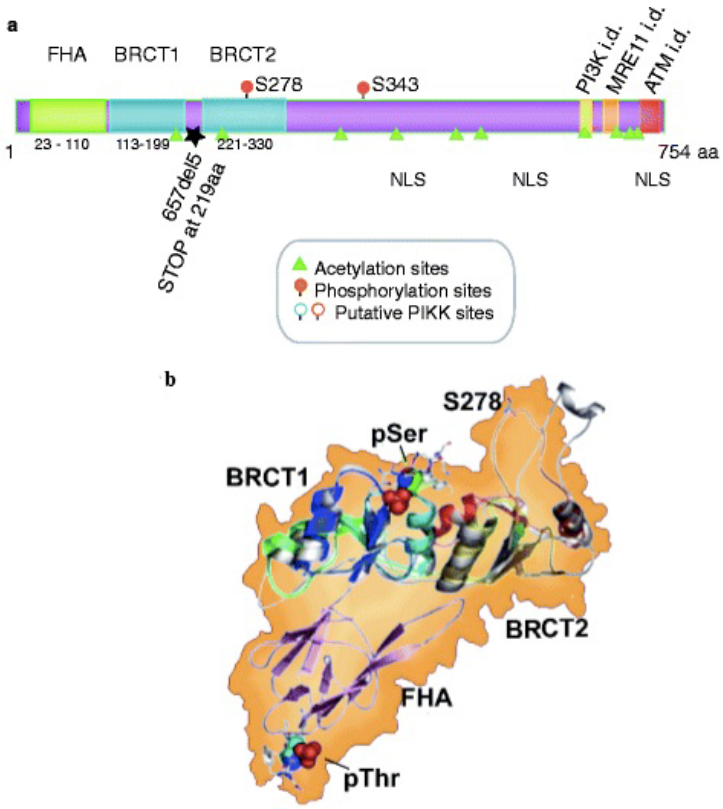

Figure 5: Structure of Nbs1

(Reproduced from Rupnik, Chromosoma, 2010)

Domain structure of Nbs1, indicated with stars are mutations found to cause Nijmegen Breakage Syndrome (NBS) (a). Three dimensional structure of Nbs1 (b)

2.4. The Baculovirus expression system

In the baculovirus expression system insect cells or larvae are used to produce heterologous protein. The strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcNPV) is often highly expressed during the late stages of infection. Heterologous proteins produced in the insect cells are mostly functional like their endogenous counterparts, since they are processed, modified and targeted to their appropriate cellular location. In contrast to other expression systems (especially prokaryotic expression systems) the baculovirus expression system has several benefits. For intracellular proteins high levels of heterologous gene expression is achieved, while the proteins are soluble and easily recovered. Hetero-oligomeric protein complex expression can be achieved by simultaneous infection with several viruses or by infection with a single virus that contains several expression cassettes. The baculoviruses have a very limited host range, what makes them safe to work with. The genome contains all genetic information needed for propagation and helper cell lines or helper viruses are not needed.

Due to the large circular double-stranded DNA genome with multiple recognition sites for restriction endonucleases, the recombinant baculovirus are constructed in two steps. First, the desired cDNA is cloned into a plasmid transfer vector downstream from a baculovirus promoter flanked by baculovirus DNA derived from nonessential loci. The heterologous expression cassette is inserted into the genome of the parent virus by homologous recombination in vivo in the insect cells. Alternatively, specifically engineered bacteria (*E.coli*) can be used for the recombination step. In this case, recombination-proficient *E.coli* containing the entire baculovirus genome are transformed with the transfer vector and the modified full length baculovirus genome is subsequently isolated from the bacteria. This can then directly be used to transfect insect cells. In this study, only the latter system was used. (Figure 6)

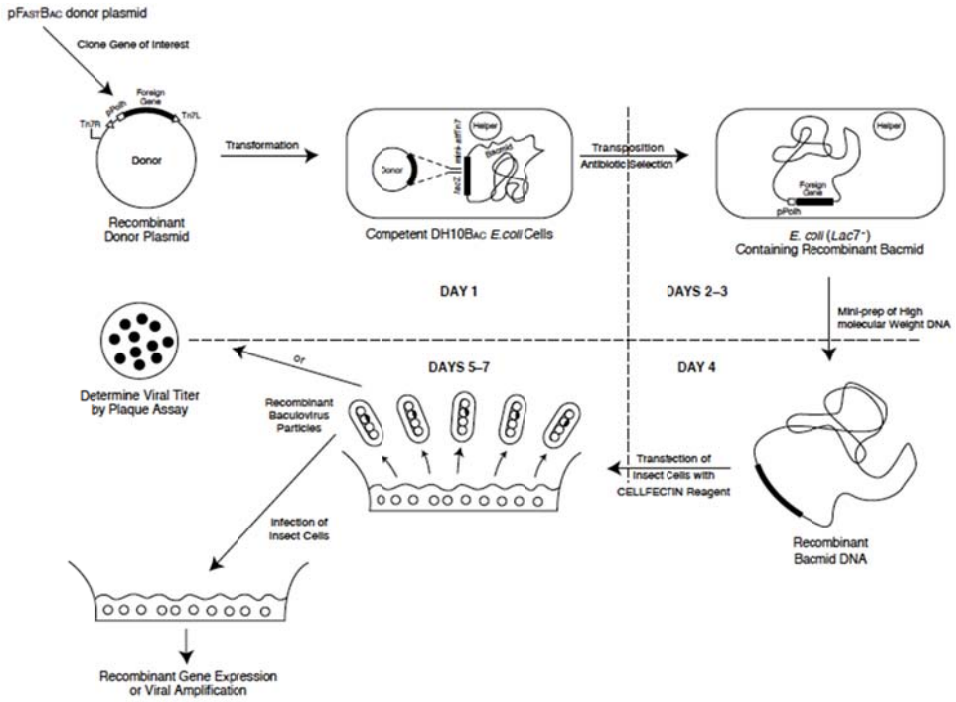

Figure 6: The Baculovirus Expression System

(Reproduced from Bac-to-Bac Manual from Invitrogen)

2.5. Electron microscopy

Single-particle electron microscopy (EM) is a good way to investigate the structure of the large complexes of the DNA repair machinery. These molecules are often dynamic and their conformation is related to their function. X-ray crystallography on its own is not able to analyse these complicated structures. In the 3D reconstruction, known X-ray structures can be fitted into the shape of the complexes in order to get a better knowledge about were specific structural elements or domains are located. But the purification of DNA repair complexes is often difficult because they are heterogeneous in their subunit content, they can have low stability and sometimes interact only with specific DNA molecules. Protein complexes can be analysed as a whole, but this makes it impossible to identify and map the regions were the proteins interact, therefore it is better to start with the analyses of the single protein (Llorca, 2007).

3. Materials and Methods

3.1. Materials

3.1.1. Antibodies

3.1.1.1. Primary antibodies

Polyclonal sheep α-Mre11 D151 (1:5000)

Polyclonal sheep α-Rad50 D149 (1:5000)

Monoclonal mouse α-alpha-tubulin clone DM1A (1:10000)	Sigma®

3.1.1.2. Secondary antibodies

Polyclonal ECL α-mouse HRP-linked sheep antibody (1:5000)	GE Healthcare
Donkey anti-goat IgG-HRP (1:5000)	Santa Cruz Biotechnology

3.1.2. Antibiotics

Ampicillin (100mg/ml in dH_2O)	Fluka® Chemie AG
Penicillin-Streptomycin (10'000 units/ml)	Invitrogen®
Kanamycin	Sigma®
Gentamicin	Sigma®
Tetracyclin	Sigma®

3.1.3. Bacteria strains

E.coli XL1 blue

E.coli DH10Bac

3.1.4. Chemical reagents

Acetic acid	Sigma-Aldrich
Acrylamid-bis 40%	Serva®
Agarose	Promega
Bacto Tryptone	BD
Bacto yeast extract	BD

Betamercapto Ethanol	Fluka®
Bromphenol blue sodium	Sigma®
Buffer 2 for restriction enzymes	New England Biolab
Buffer 4 for restriction enzymes	New England Biolab
Cellfectin	Invitrogen
Coomassie Briliant Blue R250	Fluka®
DMSO	Sigma®
ECL (two solutions, mixed 1:1)	GE Healthcare®
EDTA	Fluka®
Ethanol	Merck®
Fetal calf serum (FCS)	Gibco
Glucose	Fluka®
Glycerol	Fluka®
Glycin	Biosolve
Grace's Insect Medium	Invitrogen®
HEPES	Fluka®
Hydrochloride acid (HCl)	Merck®
IPTG	Merck®
Isopropanol	Sigma®
KCl	Fluka®
KH_2PO_4	Merck®
KOH	Merck®
Methanol	Merck®
Magnesium chlodride ($MnCl_2$)	Merck®
Nonidet P40	Fluka®
Ponceau S	Sigma®
Sodiumdodecylesulfat (SDS)	Fluka®

Sodium chloride (NaCl)	Fluka®
Sodium Hydrogenphosphate (Na_2HPO_4)	
Streptavidin, immobilized on Agarose	Fluka®
T4 DNA Ligase Buffer	Fermentas
Tris 1M	Biosolve
Tween 20	Fluka®
X-gal	

3.1.5. Enzymes

BamHI	New England Biolabs®
CIP	New England Biolabs®
EcoRI	New England Biolabs®
EcoRV	New England Biolabs®
Expand High Fidelity Enzyme (100U/µl) Mix	Roche®
HindIII	New England Biolabs®
KpnI	New England Biolabs®
SalI	New England Biolabs®
T4-DNA-Ligase	Fermentas®
XbaI	New England Biolabs®
XhoI	New England Biolabs®

3.1.6. Insect cell lines

SF21

3.1.7. Vectors

pSG5-Nbs1 (S118A)	Constructed by Lucijana Pavic
pEXPR-IBA103	IBA (BioTAGnology)
pFastBac-Nbs1-Mre11-Strep	Constructed by Lucijana Pavic

3.2. Methods

3.2.1. DNA manipulation

3.2.1.1. Agarose gel electrophoresis

Separation of digested plasmid DNA or PCR products on a 1% agarose gel and visualized the ethidium bromide staining under a UV light source.

Buffers and Medias:
1x TAE buffer
6x loading buffer
1kb Marker

Chemical Reagents:
Agarose
Ethidium bromide solution

Electrophoresis Kit:

Mini Sub DNA Cell	Bio-Rad® electrophoresis system
Power Pac 200 (at 80V)	Bio-Rad® power supplies
ChemiImager 5500	Alpha Innotech®

Gel preparations:
100ml 1x TAE buffer
1g Agarose
4µl Ethidium bromide solution

3.2.1.2. DNA-Digestion

DNA was digested for at least 2 hours at 37°C

Preparations:
15.5µl H_2O
5µl DNA (1µg/µl)

2.5µl	Buffer for Restriction enzyme (1-4)
1µl	Restriction enzyme 1
1µl	Restriction enzyme 2

To avoid self-ligation of the empty vector CIP was added for 1 hour at 37°C.

1µl	CIP	New England Biolabs

3.2.1.3. NucleoSpin® Gel-extraction and PCR clean-up

The gel extraction and PCR clean-up was done according the Macherey-Nagel protocol (protocol at a glance, rev. 03) for NucleoSpin® Extract II by using mini spin columns with silica membrane technology.

Buffers and Medias:

NT binding buffer	Macherey-Nagel®
NT3 wash buffer	Macherey-Nagel®
NE elution buffer	Macherey-Nagel®

Material:

NucleoSpin® mini spin columns

3.2.1.4. Ligation arrangements

The digested DNA fragments were ligated by T4 DNA ligase in a 15°C warm water bath overnight.

Preparations:

1µl	T4 DNA Ligase	Fermentas®
1µl	T4 Ligase Buffer	Fermentas®
3µl	Insert	
5µl	Vector	

3.2.1.5. Mini plasmid preparation (Mini Prep)

The recombinant plasmid DNA was quantified and analysed according to the following protocol:

From an overnight bacteria culture the cell pellet was collected by centrifugation (4000rpm for 10min at 4°C) and dissolved in 250µl S1 buffer. To lysate the cells 250µl S2 buffer was added and mixed gently. The protein was precipitated with 350µl S3 buffer. The mix was centrifuged at 14000rpm for 15min at 4°C. The supernatant was transferred to a new tube and mixed with 560µl isopropanol, this was centrifuged at 14000rpm for at least 30min at 4°C, the supernatant was discarded, 500µl ethanol were added and again centrifuged at 14000rpm for 10min. The supernatant was discarded and the pellet was air-dried for 2h, afterwards it was dissolved in 25µl dH_2O.

Buffers:

S1 Resuspension buffer	Macherey-Nagel®
S2 Lysis buffer	Macherey-Nagel®
S3 Elution buffer	Macherey-Nagel®

3.2.1.6. Expand high fidelity PCR System

The Expand High Fidelity PCR Kit (Roche®) includes the following contents:

- Expand High Fidelity Enzyme Mix*
- Expand High Fidelity buffer (10x) with 15mN $MgCl_2$
- Expand High Fidelity buffer (10x) without $MgCl_2$
- $MgCl_2$ 25mM Stock Solution

* Enzyme storage buffer: 20mM Tris-HCl, pH7.5 (25°C); 100mM KCl; 1mM Dithiothritol (DTT); 0.1mM EDTA; 0.5% Nonidet P40 (v/v); 0.5% Tween 20 (v/v); 50% glycerol

Additional Material:

- dNTP-Mix (deoxyribonucleosidtriphosphat mix)
- 0.2ml thin-walled PCR tubes (Axygen PCR 02-C)
- Thermal block cycler (Applied Biosystems GeneAmp® PCR System 2700)

The PCR reaction mix was prepared according the Expand High Fidelity PCR System protocol:

Volume	Reagent	Final Concentration
4.125µl	dH_2O	

2.5µl	10x Expand High Fidelity buffer (without $MgCl_2$)	
5µl	$MgCl_2$ (25mM)	
2µl	dNTP-Mix	200µM
1µl	dsDNA template	1-10ng
5µl	Forward primer	300nM
5µl	Reverse primer	300nM
0375µl	Expand High Fidelity enzyme mix	1.7U/reaction

The $MgCl_2$ amount was changed to 1, 2.5, 5µl to find the best PCR activity.

Thermal cycling:

	Cycles	Temperature	Time
Initial Denaturation	1x	94°C	5min
Denaturation		94°C	2min
Annealing	25x	55°C	30s
Elongation		72°C	2min
Final Elongation	1x	72°C	7min
Cooling		4°C	∞

3.2.1.7. Transformation of chemically competent *E.coli* cells with plasmid DNA

Transformation is a method to get plasmid DNA into a competent bacteria cell.

Thaw an aliquot competent XL1blue *E.coli* cells on ice. Pipette 2-5µl Plasmid DNA in a cooled Eppendorf, add 50µl cell suspension, tap it gently and incubate for at least 15min on ice. Heat shock the suspension 40sec at 42°C on the heating block and afterwards cool it for 1min on ice. Add 400µl liquid LB medium and culture the cells at 37°C for 30min in the shaking incubator. The cell suspension was centrifuged 2min at 14000rpm and the supernatant was discarded until 50µl were left, in this the pellet was dissolved and then spread on agar plates by small sterile glass beads. The plates were incubated overnight at 37°C.

Buffers and Medias:
LB-Medium

Bacteria cells:
E.coli XL1blue were present in the Stucki lab

3.2.1.8. Transposition

In the transposition the recombinant pFastBac donor plasmid is transformed into DH10Bac for the transposition into the bacmid.

First Luria Agar plates containing 50µg/ml kanamycin, 7µg/ml gentamicin, 10µg/ml tetracycline, 100µg/ml X-gal and 40µg/ml IPTG are made. DH10Bac competent cells are thawed on ice, 100µl of them are pipet in a round-bottom polypropylene tube and then 5µl recombinant donor plasmid is added. Incubated 30min on ice then heat shock at 42°C for 45sec, immediately chill the mixture on ice for 2min. Afterwards add 900µl S.O.C. medium and place it in a shaking incubator at 37°C for 4h. Serial dilute the cells with S.O.C. media and spread 100µl of each dilution on the plates. Incubate them for 24 till 48 hours. White colonies are then further grown in liquid media containing the antibiotics and analysed in a minipreparation.

Antibiotics	
Kanamycin	Sigma®
Gentamicin	Sigma®
Tetracycline	Sigma®

Chemicals
X-gal
IPTG

3.2.2. Protein manipulation

3.2.2.1. Determination of protein concentrations by Bradford

To determine protein concentrations of NP40 cell extracts the Bradford analysis was used. First the absorbance (595nm) of four BSA standard solutions was measured to calculate a standard

curve of absorbance. The resulting curve was used to determine the protein concentration of the samples.

Protocol:
Dilute Bio-Rad Protein-Assay Solution 1:5 in water. Prepare the samples as follows:
Standard: 20µl Standard solution + 1ml diluted Bradford solution
Blank: 20µl H_2O + 1ml diluted Bradford solution
Sample: 20µl H_2O + 2µl Lysate + 1ml diluted Bradford solution
Add Bradford solution at the end and mix it to avoid precipitation after time. Measure the adsorption at 595nm the results should be between 0.1 – 1.0.

Material:

Cuvettes (halb-mikro)	Greiner Bio One
Genesis 10UV scanning	Digitana AB thermo spectronic systems
Albumin from bovine Serum, Biochemika Fraction V	Fluka Chemie AG

3.2.2.2. SDS-PAGE (SDS-polyacrylamide gel electrophoresis)

By applying an electric field through the SDS-PAGE gel proteins are separated according their molecular weight.

Buffers:
2x SDS loading buffer
1x Stacking-Gel buffer
2x Running-Gel buffer
1x Running-Buffer
Stain
Destain

Electrophoresis Kit:

Mini-Protean Tetra Electrophoresis System	Bio Rad® vertical electrophoresis system
Power Pac 200 (15min 80V, than 150V)	Bio Rad® power supplies

Gel preparation:

	6%	8%	10%		12%	15%	Stacking gel
2x Gel buffer	2.5ml	3ml	3ml	5ml	5ml	5ml	2.4ml
40% Acrylamid-bis (w/v)	0.75ml	1.2ml	1.5ml	2.5ml	3ml	3.75ml	0.375ml
dH_2O	1.75ml	1.8ml	1.5ml	2.5ml	4ml	-	-
APS 10%	50µl	60µl	100µl	100µl	100µl	100µl	30µl
TEMED	10µl	10µl	10µl	15µl	15µl	15µl	8µl

3.2.2.3. NP40 extract

With the NP40 soluble proteins can be extracted out of the cells, and in comparison to SDS Extract the proteins are not denatured and interactions between proteins can still be analysed.

The cells were centrifuged for 1 minute at 1500rpm, then washed with PBS, afterwards they were resuspended in 150µl Extraction buffer A and incubated 10min on ice. Then they are centrifuged at 20000g for 10min. The suspension was used for further experiments.

Buffers:
Extractionbuffer A
PBS

3.2.2.4. Western blot analysis

Western Blot is a technique to transfer proteins form a SDS-Gel to a nitrocellulose membrane. The proteins can then be detected with antibodies.

The gel-membrane sandwich is prepared first with the gel located towards the black side. The blot is run in transfer buffer over night at 30V. The membrane is washed with dH_2O and afterwards stained with Ponceau S for about 10min. The part of interest is cut out and destained with TBS-T. Then it is blocked with 5% milk powder in TBS-T for 1h. The primary antibody is diluted in 2.5% milk-TBS-T (1:1000 or 1:5000) and incubated at room temperature for 4h. Next the membrane is washed 3x5min with TBS-T, afterwards the secondary antibody is added, which is diluted 1:5000 in TBS-T 2.5% milk, the membrane is incubated at RT for 1h. Then again the membrane is washed 3x with TBS-T, followed by drying and adding of the ECL-mix for 1min. The membrane is wrapped in a plastic film, put in an x-ray cartridge. An x-ray film is exposed and developed.

Material:	
Mini Trans-Blot cell	Bio-Rad blotting systems
Power Pac 200	Bio-Rad power supplies
Nitrocellulose membrane	GE Water & Process Technologies
Typon optimax	Raymed Imaging AG
Contatyp medical X-ray film	Typon Holding AG

Buffers and Medias:	
ECL (two solutions, mixed 1:1)	Amersham Biosciences
TBST	
5% TBST-milk	
Transfer buffer	

3.2.2.5. Coomassie-blue staining

Coomassie-blue staining is used to stain proteins on an SDS-Page.

The staining was done accordingly to the student course protocol TIG 15:

The gel is stained and fixed in Coomassie Brilliant Blue Staining solution about 15min. Afterwards it is rinsed with water two times and developed in destain solution.

Materials:
Staining-Box

Solutions:
Stain
Destain

3.2.2.6. ProBond resin purification

The ProBond Resin beads are used for pull downs of His-tagged protein.

Take an appropriate amount of beads and resuspend them three times in Buffer 1. Add the NP40-Extract to the beads, hold an aliquot of the extract back for further analysis, incubate the mix 30min at 4°C while rotating. After 30min take a small aliquot of the supernatant, then wash the rest 3 times with Buffer 1. Dissolve the beads in 500µl Buffer 2 to elute the bound protein

and incubate it 10min at 4°C under motion. Store an aliquot of the eluate. Continue with analyse or further purification.

Material	
ProBond Resin	Invitrogen

Buffer
Buffer 1
Buffer 2

3.2.2.7. FPLC purification

The first step of the purification was performed according to the manual on the ÄktaFPLC System. A HisTrap™ HP Column was used to trap the His-tagged Rad50. The Elution was done over a 10ml graph from Buffer A to Buffer B.

Material	
ÄktaFPLC System	Amersham Pharmacia Biotech
HisTrap™ HP Columns	GE Healthcare

Buffers
Lysisbuffer
Buffer A
Buffer B

3.2.2.8. Dialysis

Dialysis is a process to separate molecules in solution by the difference in their rates of diffusion trough a diaphragm. The Dialysis was performed according to the following protocol:

The Dialysis Cassette is hydrated in the dialysis buffer for two minutes. Afterwards the sample is added and then incubated about 30 Minutes, the buffer is changed and again incubated for 30 Minutes. Then the buffer is again changed and the cassette is incubated over night at 4°C.

Material

Slide-A-Lyzer® Dialysis Cassettes	Thermo Scientific

3.2.2.9. Streptavidin purification

With the Streptavidin immobilized on Agarose Strep-tagged protein can be purified out of a sample.

First the Streptavidin beads are equilibrated in Buffer C. Then the sample is added and incubated for one hour at 4°C. The beads are washed three times with Buffer D and afterwards the proteins are eluted with biotin. The different fractions are analysed with SDS-Gel.

Material

Streptavidin, immobilized on Agarose CL-4B	Fluka®
Biotin	

Buffers

Buffer C

Buffer D

3.2.2.10. Dynabeads purification

With the Dynabeads the Strep-tagged proteins are pulled out.

First the beads are equilibrated in Buffer C Then the sample is added and incubated for one hour at 4°C. The beads are washed three times with Buffer D and afterwards the proteins are eluted with biotin. The different fractions are analysed with SDS-Gel.

Material

Dynabeads with Streptavidin	Invitrogen
Biotin	

Buffers

Buffer C

Buffer D

3.2.3. Manipulations with bacterial and insect cell lines

3.2.3.1. Preparation of chemically competent *E.coli* cells

E.coli cells were converted into chemically competent cells to achieve better transformation efficiency according to the following protocol:

All the tubes and solutions were chilled and it is recommended to work in the cold room. 1ml XL1blue *E.coli* strain was incubated in 200ml S.O.C. medium at room temperature overnight. The cells were grown to an OD600 as close to 0.45 as possible, the cultures were split into sterile falcon tubes and place on ice for 10min, then they were centrifuged 15min at 3500rpm at 4°C, the supernatant was carefully discard. The pellet was resuspended in 64ml HTB and placed on ice for 10min. After centrifugation at 3500rpm for 15min at 4°C, the supernatant was carefully discard and the pellet resuspended in 16ml. Afterwards 1.2ml filter sterilized DMSO was added slowly. Then the suspension was aliquoted in pre-cooled Eppendorf tubes (200µl per tube) and shock-frozen in liquid nitrogen. The aliquot is stored at -80°C.

Material:

Cuvettes (halb-mikro)	Greiner Bio One
Genesis 10UV scanning	Digitana AB thermo spectronic systems
Sorvall RC 3C Plus	Sorvall centrifuges
Sorvall HRL6	Sorvall rotors
Nalagene 1000ml tubes	

Buffers and Medias:

S.O.B. Medium

S.O.C. Medium

HTB buffer

Chemical Reagents:

DMSO

3.2.3.2. Transfection

Transfection is a method to bring foreign DNA into cells of higher eukaryotes.

The instruction manual Bac-to-Bac® Baculovirus Expression Systems from Invitrogen was used. Seed $9x10^5$ cells per well in 2ml media containing Penicillin/Streptomycin and FCS. Let the cells attach for about 1h. Prepare Solution A containing 5µl of min-prep bacmid DNA in 100µl media without antibiotics and Solution B containing 6µl CellFECTIN in 100µl media without antibiotics. The two solutions are mixed and incubated for about 30min. Wash the cells with media without antibiotics. Then add 0.8ml media to the mixed solutions and overlay the cells. Incubate it for 5h at 27°C. Remove the transfection mix, add 2ml media with antibiotics and incubate it for 72h at 27°C.

Buffers and Medias
Graces Insect Media w/o Penicillin/Streptomycin + FCS
CellFECTIN

Cell lines
SF21

Materials
6-well plate
27°C Incubator

3.2.3.3. Virus titration

Virus titration is a method to determine the efficiency of the produced virus. The titration was done according to the following protocol:

The cells were scratched off the plate and filled in a tube. With the "Neubauer counting chamber" the amount of cells per Millilitre was determine. If necessary the volume was adjusted to $20x10^5$ cells per 2ml media, this was then seeded on a 6-well plate and incubated at 27°C for 1h. Afterwards the media was aspirated and 500µl new media was added. The virus was added in different amounts and the plate was again incubated at 27°C for 1h. After one hour 1.5ml media was added and the plate incubated 2-3 days at 27°C. At the end of the incubation the infected cells can be processed further.

Buffers and Medias
Graces Insect Media w/o Penicillin/Streptomycin + FCS

Cell lines
SF21

Virus
Baculovirus with Mre11 cDNA
Baculovirus with Rad50 cDNA

Material
6-well plate
27°C incubator

3.2.3.4. Virus amplification

Virus amplification is a technique to amplify the virus stock.

The cells were plated out, infected with the reproductive virus, incubated for two days and harvested. The remaining cells were centrifuged and the supernatant transferred to a new tube.

Buffers and Medias
Graces Insect Media with Penicillin/Streptomycin + FCS

Cell line
SF21

Virus
Baculovirus Mre11

Material
100mm plates
Falcontubes

4. Goals of the thesis

4.1. Expression and purification of differentially tagged Mre11/Rad50 heterodimer from baculovirus-infected insect cells

Based on preliminary results using electron microscopy (EM) to monitor structural changes in the MRN complex upon binding to MDC1 our group started a collaboration with Prof. Oscar Llorca in Spain. Oscar Llorca is an expert in structure determination of large multi-subunit DNA repair complexes by EM reconstruction. Previously, the former technical assistant Lucijana Pavic purified MRN and MR subcomplexes from Baculovirus infected insect cells and these purified proteins were sent to Spain for analysis. The data revealed that the MR heterodimers were present as monomers, multimers and protein aggregates. Since both Rad50 and Mre11 were tagged with a His6-affinity tag, it was not possible to separate monomeric proteins and protein aggregates from heterodimeric Mre11/Rad50 complexes. Therefore, in order to overcome this problem, the aim of this project was first to generate Streptavidine-binding peptide (Strep)-tagged Mre11. This could then be co-expressed with His-tagged Rad50 in Baculovirus-infected insect cells. Subsequently, tandem purification over Nickel-NTA columns and Streptavidin beads could be used to purify the MR dimers.

5. Results

5.1. Tandem purification of Mre11/Rad50-dimers using the Baculovirus expression system

5.1.1. Virus production

Preliminary data from our lab showed that Mre11 and Rad50 when co-purified over a His-affinity column were present in monomers, dimers and aggregates. To isolate pure dimers of Mre11 and Rad50, we sought to exchange the Mre11-tag from His to Strep, while the Rad50 His-tag was left unaltered. Since our former technician L. Pavic had already constructed a bacmid containing the Strep-tagged full-length Mre11 cDNA, this construct was used directly for recombinant virus production. The bacmid DNA, containing the sequence of Mre11-Strep, was first transfected into SF21 insect cells to produce the Baculovirus. The transfection led to six different virus stocks (1A-1C, 2A-2C). These viruses were then tested for their Mre11 expression capacity through an infection of the SF21 cells. The cells were collected 3 days post infection and the proteins were extracted with a NP-40 extraction buffer and loaded on a SDS-Gel, Bradford protein concentration test was used to load equal amounts of total protein in each lane. Mre11 expression was analysed by western blotting, using a sheep anti human Mre11 polyclonal antiserum. All six virus stocks showed a similar expression of Mre11. Some degradation products could be seen on the blot (lower bands in Figure 7), probably because no protease inhibitors were used in this extract.

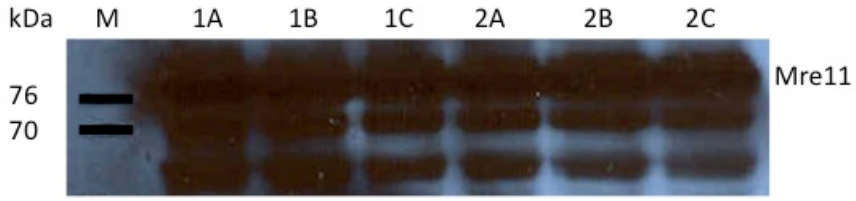

Figure 7: Mre11 is expressed in all produced virus stocks. α-Mre11-Ab. Exposure: 10 sec.

Next, a titration of one Mre11 virus stock (1A) was performed to test the best concentration of virus for the expression. In parallel, the Rad50 virus produced by L. Pavic was also titrated in the same way. The extraction was again done with NP-40 extraction buffer. While the Rad50 virus showed a high titer (Figure 8), the Mre11 virus concentration was low (Figure 9) and thus, it was decided to amplify it. For the amplification, SF21 cells were infected with recombinant Mre11

baculovirus and the supernatant was harvested after 48 hours. The thus amplified Mre11 virus was again titrated and this time, showed a far higher titer (Figure 10).

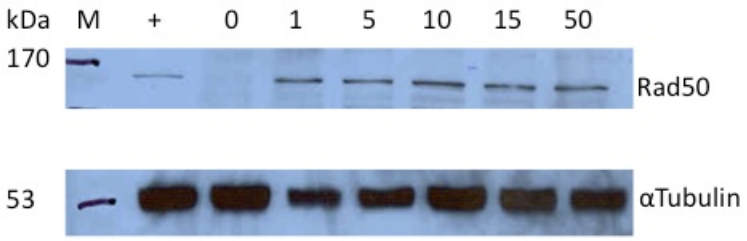

Figure 8: Rad50 titration. x µl were used for cell infection. As positive control an U2OS cell extract was used. α-Tubulin was used as loading control. Exposure: 30 sec.

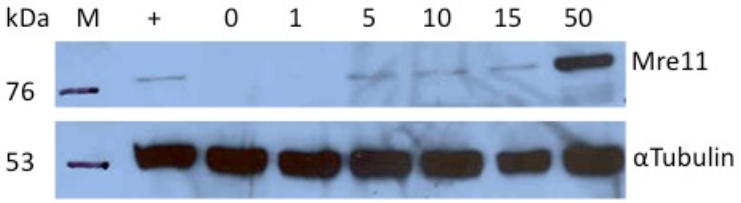

Figure 9: First Mre11 titration. x µl virus were used for cell infection. As positive control an U2OS cell extract was used. α-Tubulin was used as loading control. Exposure: 30 sec.

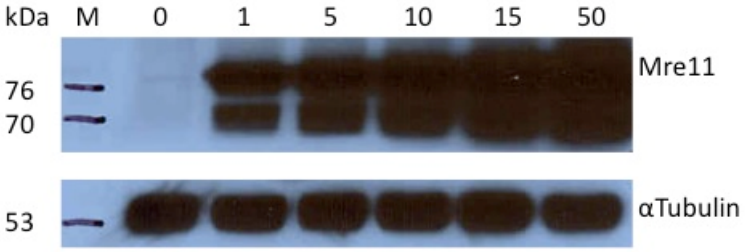

Figure 10: Second Mre11 titration after amplification. x µl virus were used for cell infection. α-Tubulin was used as loading control. Exposure: 30 sec.

5.1.2. Protein purification

With these results we decided to perform a pilot expression in order to test the binding efficiency of the MR complexes to the Nickel-NTA resin and the Streptavidin beads, respectively. SF21 cells were co-infected with the same amount of Mre11 and Rad50 Baculovirus. The NP-40 cell extract was then incubated with Nickel-NTA resin. The resin was washed 3 times with Buffer 1 and the bound proteins were eluted with 500µl Buffer 2. Subsequently, the eluted fraction was

dialyzed and incubated with the second affinity matrix (Streptavidin immobilized on agarose). The Streptavidin beads were washed and bound protein was eluted with SDS loading buffer. The different samples were then analysed by SDS-PAGE and Coomassie blue staining or Western blotting. The results revealed that some of the protein could bind to the first Nickel-NTA resin (see Figure 11, lane EL1). However, a lot of protein was found in the flow through, which basically indicates that most of the protein did not bind efficiently to the affinity beads. In the second purification step, no proteins were found in the elution, but everything was found in the supernatant (see Figure 11, lane SN2 and EL2), indicating that no MR heterodimeric complexes bound to the Streptavidin beads.

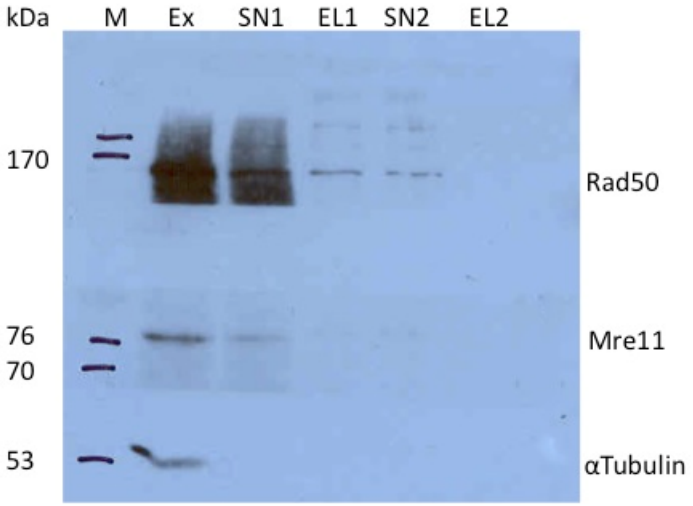

Figure 11: Test expression and purification. NP-40 Extract (Ex) was purified over ProBond Resin beads, after incubation a supernatant probe (SN1) was taken to see the binding efficiency. After 3 wash steps the protein was eluted (EL1), this eluat was used to purify over Streptavidin beads. Again the supernatant (SN2) was taken after incubation. The beads were then eluted (EL2), which showed that the whole protein was lost and found in the supernatant. Exposure time: 30 sec.

After the pilot expression a larger number of plates were co-infected with recombinant Mre11 and Rad50 baculoviruses. The extraction procedure was adjusted to the conditions that are required for the ÄktaFPLC, so Triton 100 was used instead of NP-40. A HisTrap^{TM}HP Column was used to capture the His-tagged Rad50 monomers, as well as the MR dimers. The proteins were then eluted with a 10 ml linear gradient of Imidazole (20 mM – 200 mM) and the purified protein factions were analysed by SDS-PAGE (Coomassie staining) and by Western blotting using sheep anti human Rad50 and Mre11 polyclonal antisera, respectively. The results revealed

that only very little protein bound and eluted from the column. Nevertheless, the purification was continued.

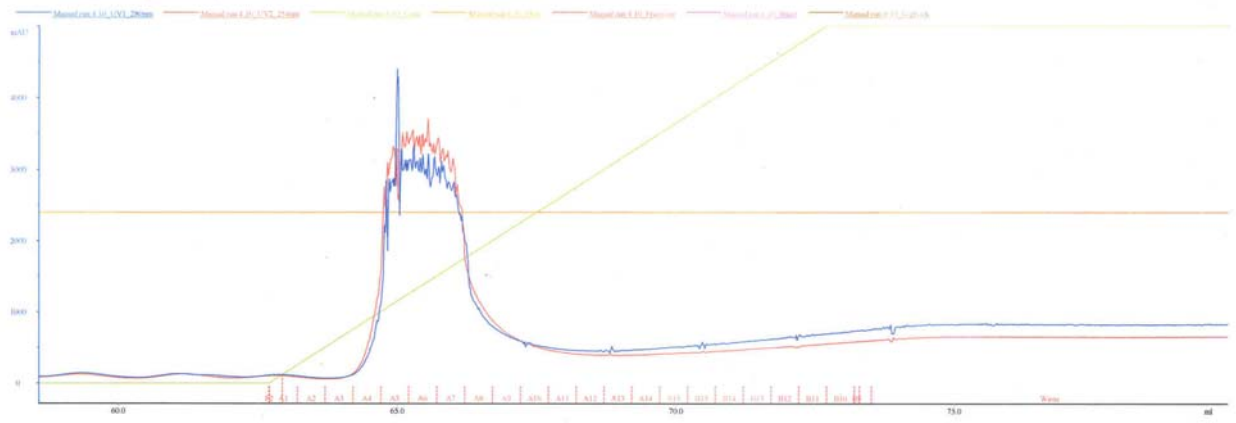

Figure 12: ÄktaFPLC elution graph. UV 280nm: blue line and UV 254nm: red line rise when the protein is eluted. Increasing concentration of Imidazole buffer: green line.

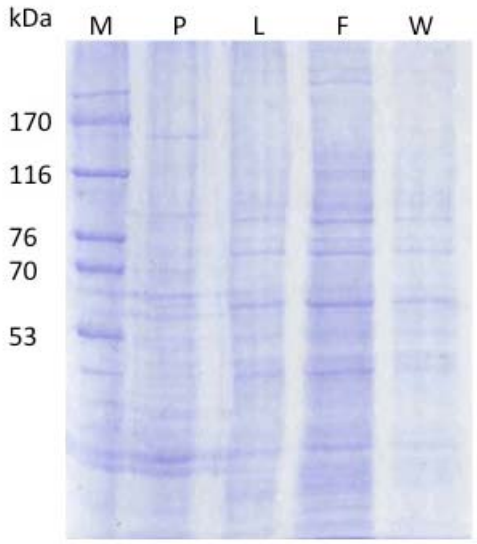

Figure 13: Fractions after ÄktaFPLC purification of SF21 cell extract over His-trap column. From the Lysate too little was loaded to see the Rad50 and Mre11 bands. The binding capacity of the column seems to be good since neither Mre11 nor Rad50 can be detected on the Coomassie. Marker (M), pellet (P), lysate (L), flow through (F), wash (W)

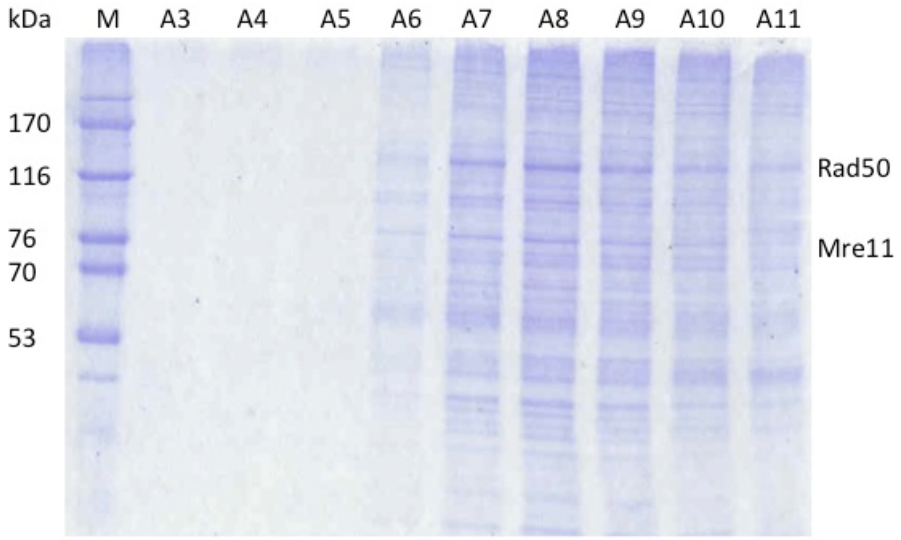

Figure 14: Eluted fractions after ÄktaFPLC purification of SF21 cell extract over His-trap column. The Rad50 concentration increases from fraction A6 on to A9 and decreases again. Mre11 shows a similar behaviour. Marker (M), Fractions (A3-A11).

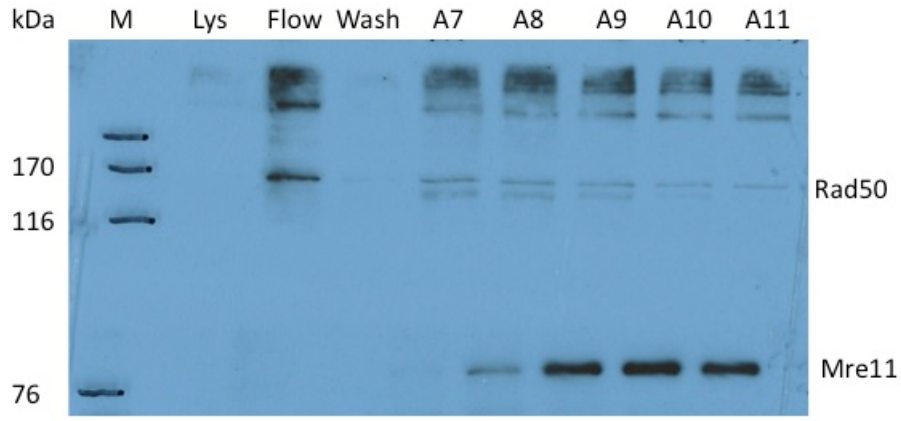

Figure 15: Western blot analysis of the ÄktaFPLC purification of SF21 cell extract over His-trap column. From the Lysate too little was loaded to detect the protein. In the flow through a part of the Rad50 was lost. Rad50 shows a slight accumulation in the fractions A7 – A9, afterwards the concentrations decreases. Mre11 is pulled down with the latter fraction A9 –A11. Marker (M), Lysate (Lys), Flow through (Flow), Wash (Wash), Fractions (A7 –A11). Exposure time: 30sec.

The fractions A7 – A9 were pooled for the next purification step. This pool was then dialysed with a dialysis cassette against buffer d to remove the Imidazole and to adjust the salt concentration for the next purification step. First, we tried to purify the dialysate over Streptavidin immobilized on agarose. But the SDS-PAGE analysis and a Western blotting revealed that neither in the elution nor on the beads Mre11 and Rad50 protein was detectable, only in the flow through

fraction the proteins appeared. (Figure 16, lane "Flow") Since Streptavidin-coated Dynabeads were available in the laboratory, the supernatant was tested if the protein would bind to these beads. But again the protein did not bind to the beads and was found completely in the flow through (data not shown).

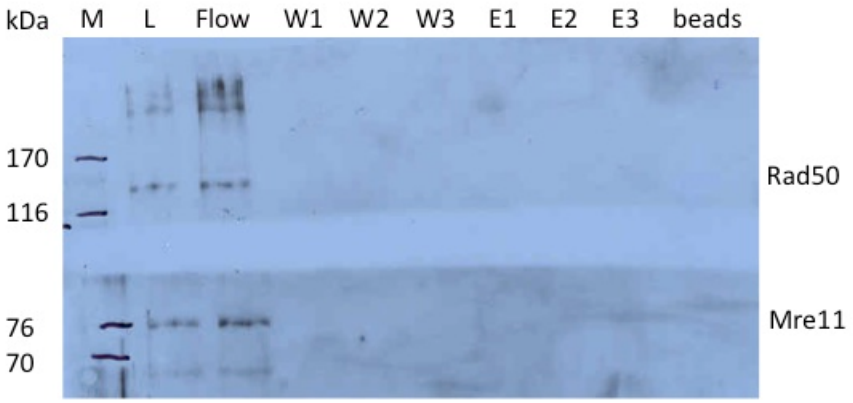

Figure 16: Western Blot of Strep tag purification over Streptavidin, immobilized on agarose. The proteins were lost in the Flow through; this indicates that the protein could not bind to the beads. Marker (M), lysate (L), flow through (Flow), washing steps (W1 –W3), elate (E1 – E3). Exposure time: 1 min

Why did the recombinant Mre11 and Rad50 proteins not bind to the beads? First, the protein expression level could have been insufficient and thus, the protein concentration too low to bind efficiently to the beads. Alternatively, it was also possible that the sequence of the Strep tag was not correct, which would also interfere with protein binding to the Streptavidin beads.

To test these two possibilities, the protein expression was repeated and at the same time, the Mre11-Strep transfer vector was sent for sequencing. For this second large-scale expression a larger number of plates were co-infected with Mre11 and Rad50 recombinant Baculoviruses. The extraction was done in the same way as before. The first purification step was again carried out over the ÄktaFPLC system with a HisTrap^{TM}HP column. The first elution didn't seem to work because no protein could be detected in the elution fractions, neither by UV absorption nor by the Bradford protein concentration assay. It was discovered that one tube of the B-pump of the Äkta system was loose. After repairing, the elution was repeated with a gradient of Imidazole and proteins in the elution fractions were detected. Nevertheless, the UV absorbance curve of the Äkta system looked odd (Figure 17), which could later be explained by a defective UV-lamp that was detected upon maintenance of the Äkta-FPLC system. The fractions with the highest protein concentration (according to the Bradford assay) were analysed by SDS-PAGE and Western blot-

ting. This time SDS-PAGE (Coomassie blue stain) results showed a better purification efficiency than in the previous run and the best three fractions were pooled for dialysis (Figure 18 and 19). Unfortunately, at the same time the sequence report from Microsynth revealed that the Mre11-Strep construct was faulty. The expression cassette contained only a short tag with a STOP codon instead of a full length Strep-tag, which would explain why the recombinant Mre11 proteins would not bind to the Streptavidin beads. Because of this result, the protein production was stopped.

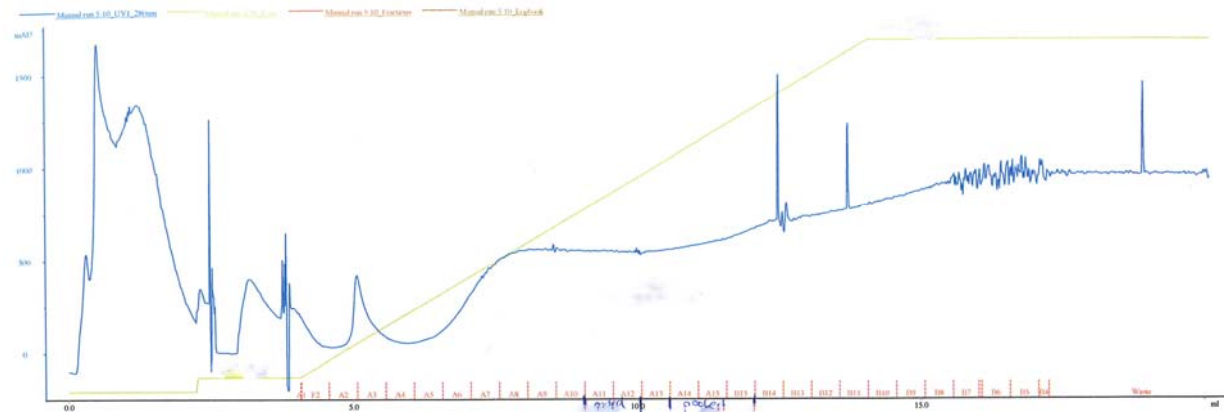

Figure 17: Second Elution with the ÄktaFPLC of SF21 cell extract over a His-trap column. The first part of the UV 280nm spectrum looks different because there the ÄktaFPLC was adjusted to start again with the purification. While the fractions were eluted the UV 280nm spectrum was rising but no peak like normal appeared. UV 280nm (Blue line), Concentration of Imidazole Buffer (Green line).

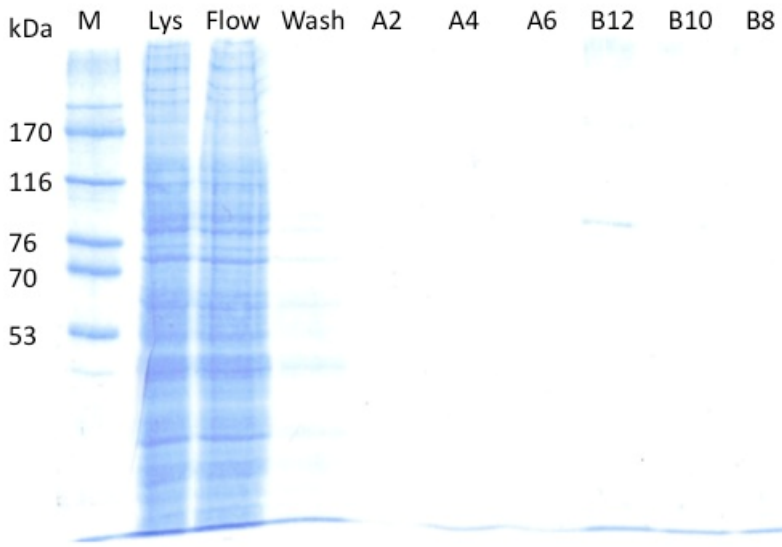

Figure 18: Fraction analyses after ÄktaFPLC purification with His-trap column. Almost no Rad50 or Mre11 protein bands are visible. Marker (M), lysate (Lys), flow through (Flow), wash (Wash), fractions (A and B)

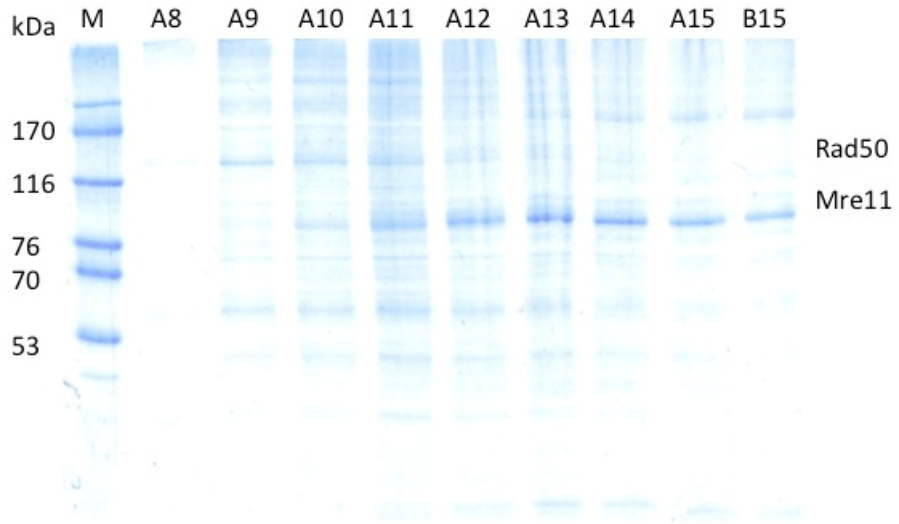

Figure 19: Fraction analyses after ÄktaFPLC purification with His-trap column. The Rad50 was eluted visibly after fraction A9 and decreased again after A12. The Mre11 is traceable from Fraction A9 till B15. Marker (M), Fractions (A – B).

5.1.3. Construction of a new C-terminally-tagged Mre11-Strep transfer vector

In order to generate a transfer vector containing the full length Mre11 cDNA with an intact C-terminal Strep-tag, the same cloning strategy was used that had already been used by Lucijana Pavic. This cloning strategy included three steps: since no baculovirus transfer vector containing a C-terminal Strep tag is commercially available, the cloning was started with the pEXPR-IBA103 vector (IBA BioTAGnology) containing a C-terminal Strep-tag. The multiple cloning site (MCS) a Strep-tag sequence of pEXPR-IBA103 (Figure 20) was transferred into the pFast-Bac1 vector of the Bac-to-Bac Baculovirus expression system from Invitrogen. In order to make the MCS of pEXPR-IBA a bit longer (which would facilitate the isolation of the DNA from an agarose gel) an insert with matching restriction sites from the vector pSG5-NBS1 was cloned into pEXPR-IBA. Both pSG5-NBS1 and pEXPR-IBA were digested with XhoI restriction enzyme. The products of this reaction were separated on a 1% agarose gel and the bands of interest were cut out of the gel and purified with the NucleoSpin® Gel-Extraction Kit. Vector and insert were then ligated with T4 DNA ligase. The ligation reaction was afterwards transformed into XL1blue chemically competent *E.coli* cells. The plasmid DNA was then isolated by a miniprep-aration and test digested with XhoI in order to verify the ligation success (Figure 21).

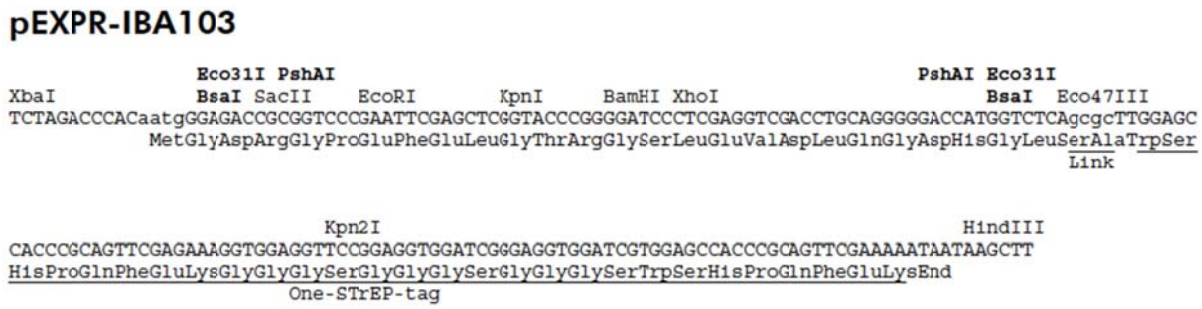

Figure 20: The pEXPR-IBA103 vector and multiple cloningsites

(Reproduced from One-STrEP Kit manual from IBA BioTAGnology)

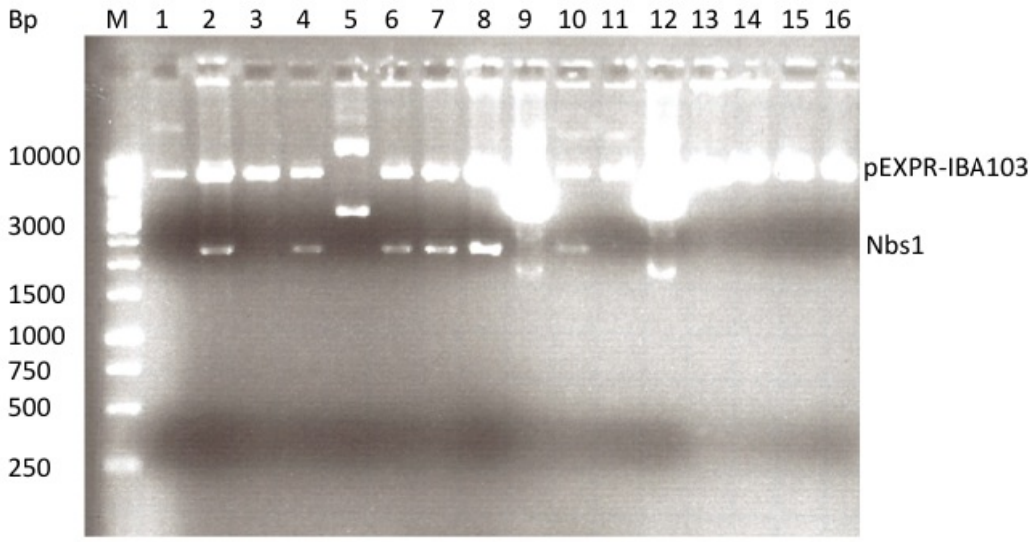

Figure 21: Test digestion of minipreparation pEXPR-IBA103-NBS1 with XhoI. The vectors 2, 4, 6, 7, 8 and 10 have the Nbs1 insert. Marker (M), minipreparations (1-16).

The resulting pEXPR-IBA103-NBS1 (7) was then used to transfer the Strep-tag into the pFast-Bac vector (Figure 22). For this both vectors were cut with the BamHI and HindIII restriction enzymes. The fragments were separated on a 1% agarose gel and processed as described above. The test digestion to verify successful ligation was performed with BamHI and HindIII (Figure 23).

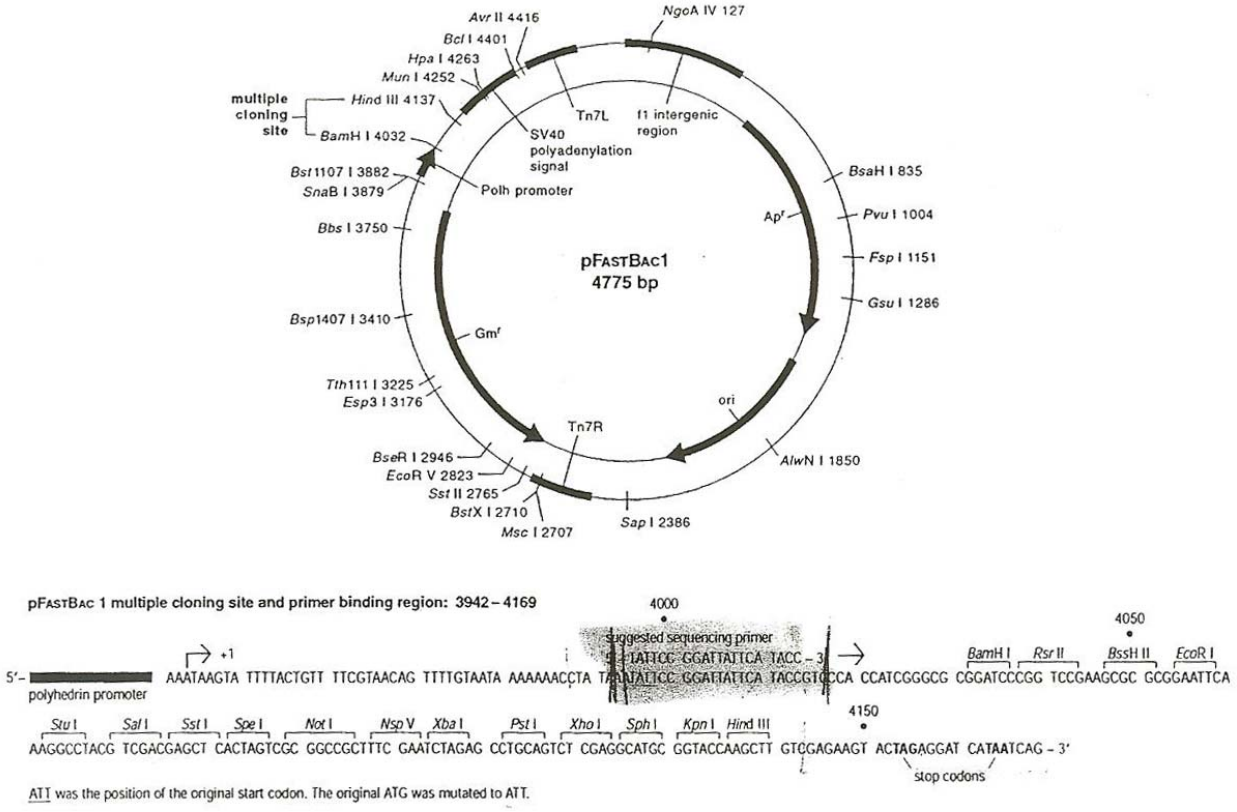

Figure 22: pFastBac1 vector map and multiple cloning sites

(Reproduced from Bac-to-Bac manual from Invitrogen**)**

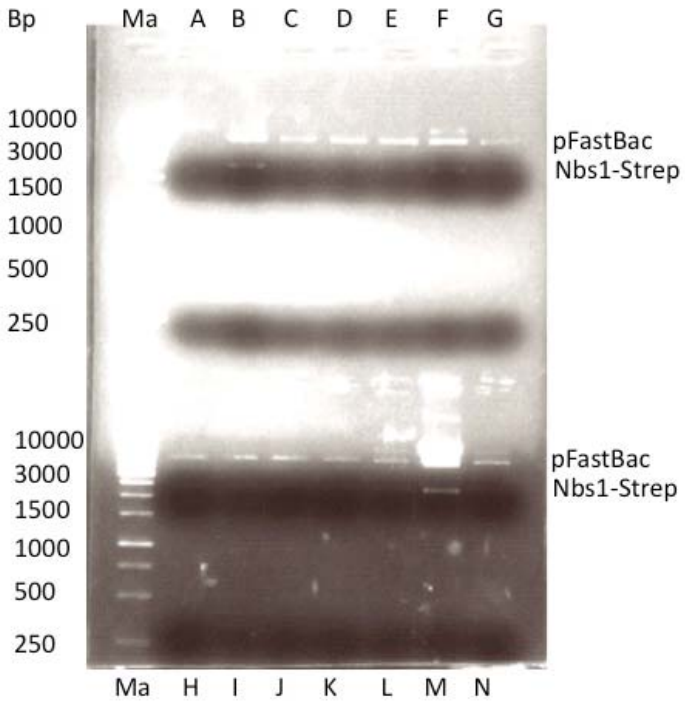

Figure 23: Test digestion of pFastBac-NBS1-Strep with BamHI/HindIII The vectors B, C, D, E, I, J, K have the Nbs1-Strep insert. Marker (Ma), minipreparations (A-N)

The results in Figure 23 showed that the ligation was successful and thus, one clone (B) was chosen to perform the last step. In this step, full length Mre11 cDNA lacking the stop codon was excised from the pMGS3 vector with SalI and subsequently ligated into the pFastBac-Nbs1-Strep vector where the Nbs1 insert was cut out with XhoI (note that XhoI and SalI produce compatible ssDNA overhangs). Again, the same procedure was used to ligate the vector and the insert. For the control digestion, BamHI and EcoRI were used, because EcoRI cuts also within the open reading frame and thus, the orientation of the Mre11 cDNA could also be tested. After several attempts the right fragment sizes were obtained (Figure 24). Two of these positive clones 4 and 10 were sent for sequencing.

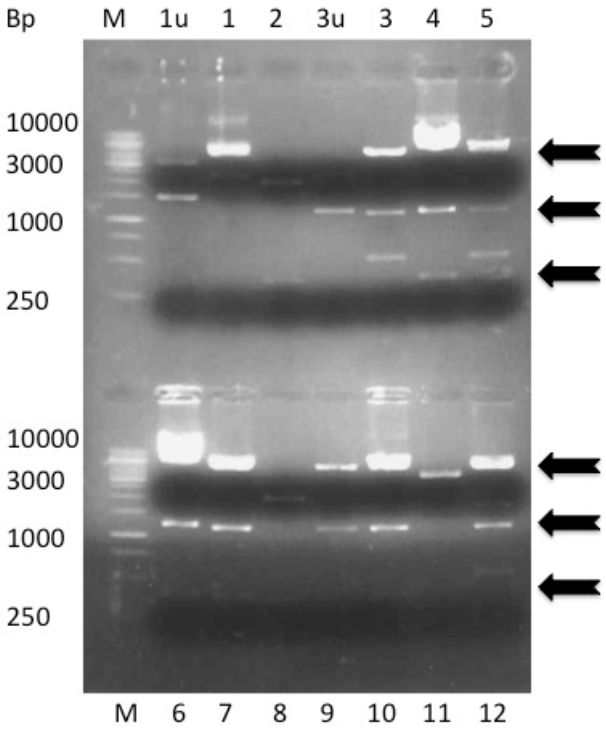

Figure 24: Test digestion of pFastBac-Mre11-Strep with BamHI/EcoRI. The vector 4, 6, 7, 10 have the predicted molecular weight (←). The two vectors were included undigested (*u) as negative controls. Marker (M), minipreparations (1-12).

Unfortunately, the sequencing results showed that none of the constructs had the right sequence. Again, both the sequenced clones showed the same truncated C-terminal tag that we already observed in the construct prepared by Lucijana Pavic. We concluded that this cloning strategy did not yield the expected results and decided to use another strategy.

To minimize the error rate the first two steps of the above strategy were combined to a one step cloning procedure. The Strep-tag was directly excised of the vector pEXPR-IBA with BamHI and HindIII. Due to the small weight of the fragment it was purified on a 2% agarose gel. The Strep-tag fragment was then ligated into the pFastBac vector, which was also digested with BamH1/HindIII. XhoI and EcoRV digestion was used to verify the ligation success. The predicted fragment sizes were 3500 and 1200 kb, respectively. The results in Figure 25 revealed that this ligation worked well and all selected clones were positive in the test digestion. To verify the result, three samples were sent for sequencing.

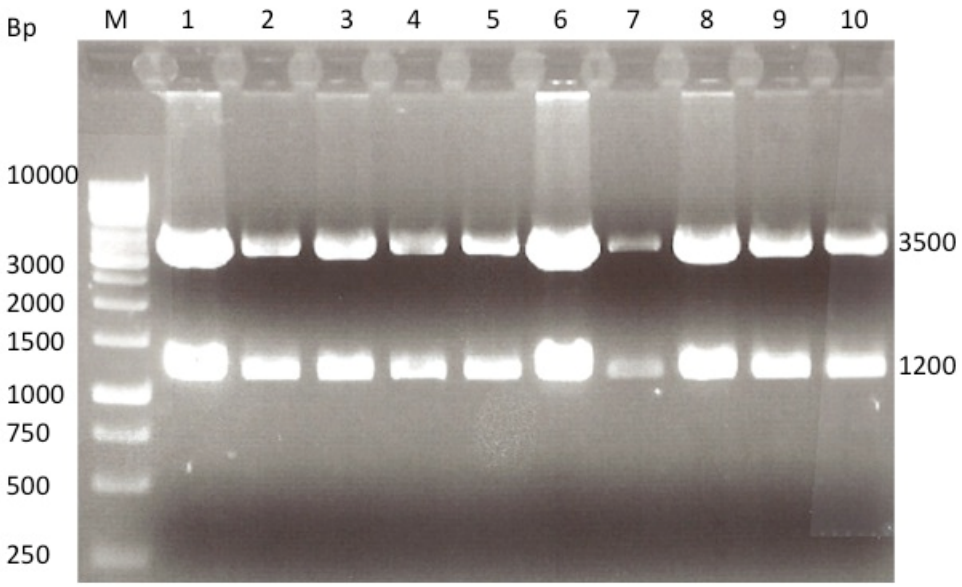

Figure 25: Test digestion of pFastBac-Strep Minipreparation with XhoI and EcoRV. All colonies were positive with the calculated piece size 3500 and 1200. Marker (M), minipreparations (1-10).

The sequencing report showed that all selected clones had the right sequence. Therefore, the second step of the cloning was done with this new vector. The modified pFastBac vector was digested with XhoI and purified. As described above, the Mre11 cDNA was excised from pMGS3 with SalI and ligated into the modified pFastBac. Unfortunately, the test digestion showed that all the clones contained the insert in the wrong orientation (3' to 5') (Figure 26).

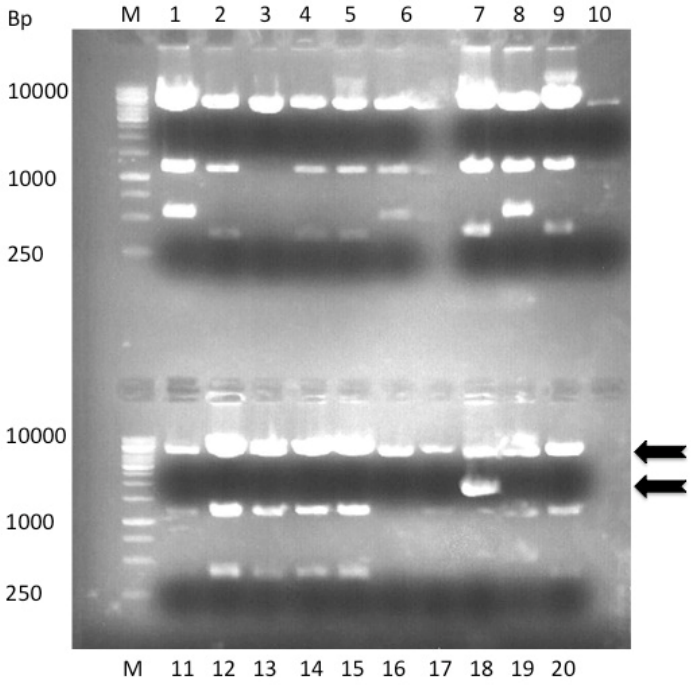

Figure 26: Test digestion of pFastBac-Mre11-Strep Minipreparation with BamHI and EcoRI. Vector 18 showed a similar hight like predicted, but its running pattern indicated that it might be empty vector. Marker (M), minipreparations (1-20).

Therefore, we decided to change the cloning strategy completely. Instead of excising the Mre11 cDNA from pMGS5 by using already existing restriction sites, we decided to insert a novel 5' (upstream) restriction site by PCR. This restriction site (KpnI) is different from the 3' (downstream) site and thus, the chance to isolate clones that contain the insert in the right orientation would increase significantly.

In the first step the Strep-Tag was cut out of the pEXPR-IBA103 vector with HindIII/EcoRI to ligate it into the pFastBac1 vector. Since the EcoRI didn't cut properly, test digestions were set up to find a working lot (data not shown). Since the test digestions used up a lot of pFastBac1 a Midi was done to get more DNA. Afterwards both vectors were again cut with EcoRI/HindIII (Figure 27/28).

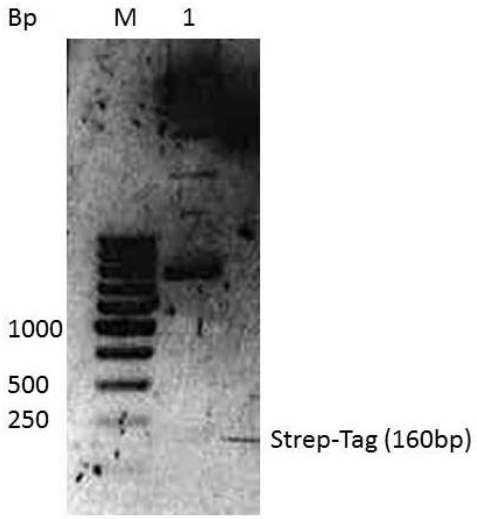

Figure 27: Digestion of pEXPR-IBA103 (1) with HindIII and EcoRI. Strep-Tag at 160bp faint visible. Marker (M).

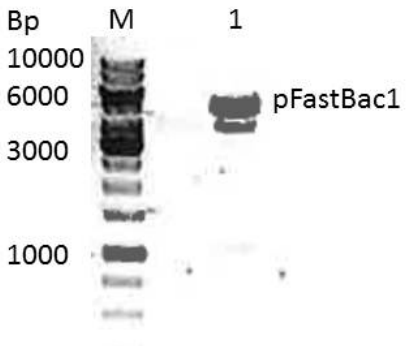

Figure 28: Digestion of pFastBac1 (1) with HindIII and EcoRI. Marker (M).

The fragments were gel purified and afterwards ligated and 10 Minipreparation with the new pFastBac1-Strep vector were done. These were digested with EcoRV and XbaI. The Minipreparation 6, 8, 9 and 10 showed the right digestion pattern (Figure 29), they were sent in for sequenzing. 6, 9 and 10 were positive, 8 couldn't be read. The pFastBac1-Strep vector 6 was used for a Midi to get a higher amount of DNA.

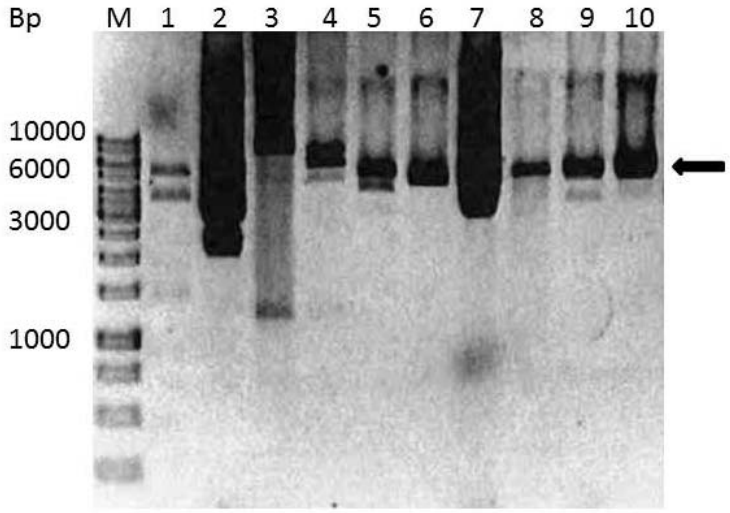

Figure 29: Testdigestion of the Minipraparation of pFastBac1-Strep vector with EcoRV and XbaI. Marker (M), Minipreparation (1-10).

In parallel the Mre11 was modified to contain a KpnI side. For this a PCR with the primers up_Mre11_Kpn1 and rev_PCR2.1_TOPO was run. The final product was then cut with KpnI and XhoI (Figure 30).

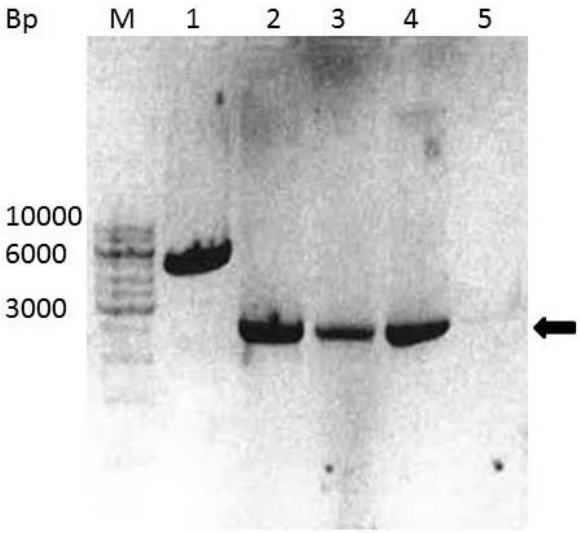

Figure 30: Mre11-Kpn1 PCR. The PCR product was lost in the gel purification. Marker (M), pFastBac1 vector (1), PCR 1.5µl $MgCl_2$ (2), PCR 3µl $MgCl_2$ (3), PCR 5µl $MgCl_2$ (4), PCR gel purification (5)

The pFastBac1-Strep vector 6 was then cut with KpnI and XhoI, while the Mre11-KpnI was cut with KpnI and SalI. The resulting products were gelpurified and then ligated. The ligation was tested in 20 Minipreparations that were testdigested with KpnI and HindIII. From the positive clones (1000bp/1200bp) 2, 4 and 7 were sent in for sequenzing (Figure 31). All three had the correct sequence and clone 2 was used for the further experiments.

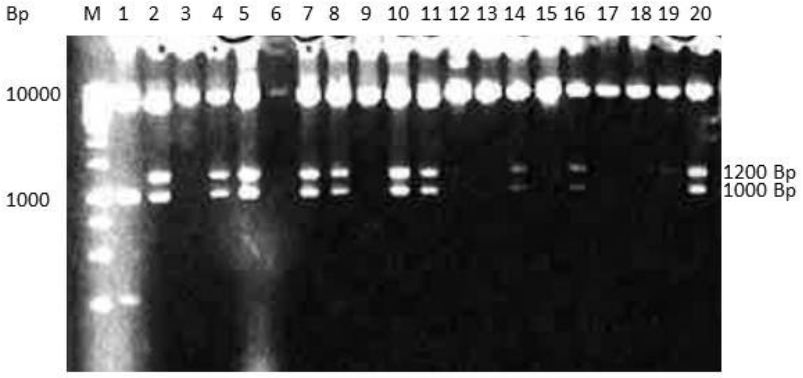

Figure 31: Testdigestion pFastBac-Mre11-Strep with KpnI/HindIII. The Minipreparations 2, 4, 5, 7, 8, 10, 11, 14, 16, 20 are positive. Marker (M), Minipreparations (1-20).

The recombinant pFastBac-Mre11-Strep was then used to transform the DH10Bac *E. coli* strain for transposition of the Strep-tagged recombinant Mre11 cDNA into the bacmid. For this the transformed bacteria were streaked out on Lb-agar plates containing kanamycin, gentamicin, tetracycline, x-gal and IPTG. A lot of colonies and satellites grew and it was difficult to distinguish between the blue and the white colonies. Therefore eleven colonies were picked for further analysis by PCR. This PCR analysis revealed that bacmid clones 5 and 7 contained the Strep-tagged Mre11 cDNA (Figure 32). Therefore bacmid clone 5 was used for the recombinant virus production.

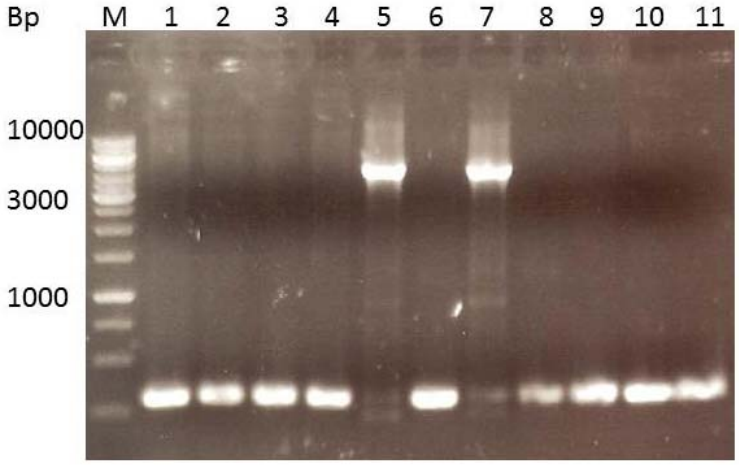

Figure 32: Control PCR of the bacmid DNA. Bacmid clones 5 and 7 are positive. Marker (M), Minipreparations (1-11).

5.1.4. Protein purification with the new vector

The bacmid DNA was transfected into the SF21 insect cells. 3 different virus stocks were acquired and the remaining cells were tested for Mre11 expression. A weak band was visible on a Coomassie blue stained gel with an apparent molecular mass that would fit that of Mre11 (Figure 33).

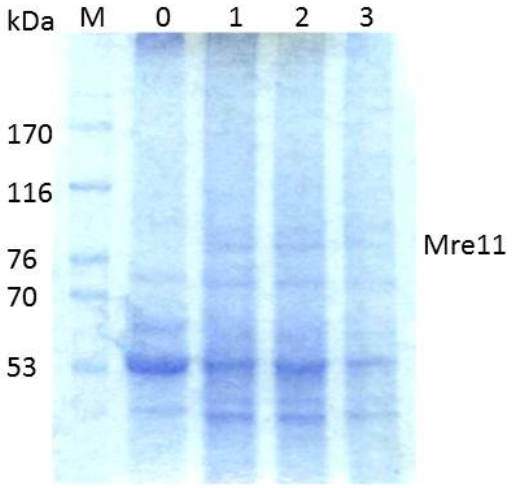

Figure 33: Mre11-Strep transfection coomassie blue staining. Marker (M), negative control (0), bacmid transfected (1-3).

Next, SF21 cells were infected with the three clones combined to amplify the virus. With the amplified virus stock, co-infection was performed with Mre11-Strep and Rad50-His viruses. Proteins were extracted with NP-40 buffer and purification was done over Ni-NTA resin and Streptavidin beads. Unfortunately, neither purification resulted in detectable amounts of purified protein on a Coomassie blue stained SDS gel (Figure 34).

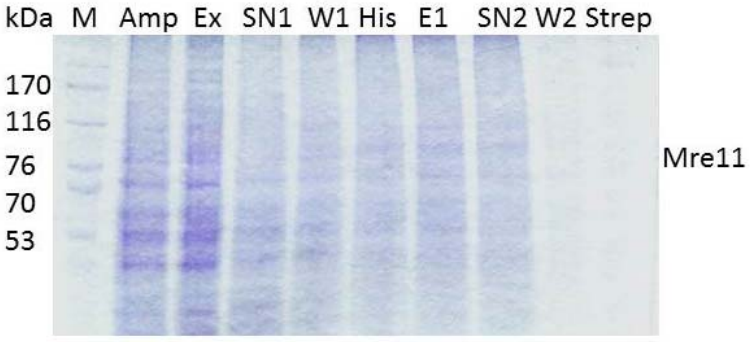

Figure 34: Amplification of Mre11-Strep and co-infection with Rad50-His. Faint band of Mre11 visible in the amplification and the extract. Marker (M), Amplification (Amp), Extract (Ex), Supernatant (SN), Wash (W), His-beads (His), Eluat (E1), Streptavidin beads (Strep).

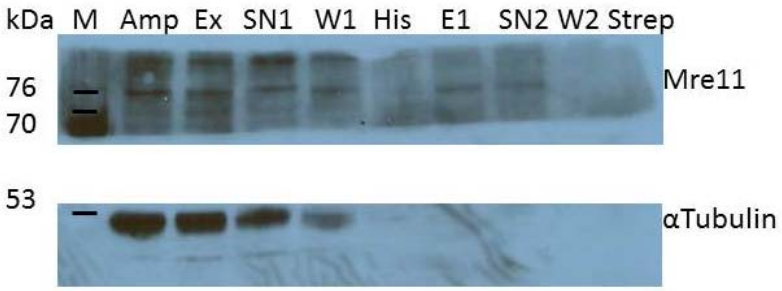

Figure 35: Amplification of Mre11-Strep and co-infection with Rad50-His. The Rad50 antibody did not work, therefore the data is not shown. Mre11 binds not on the Streptavidin beads and is all lost in the supernatant 2. Marker (M), Amplification (Amp), Extract (Ex), Supernant (SN), Wash (W), His-beads (His), Eluat (E1), Streptavidin beads (Strep).

Therefore, in order to test if the expression was successful and the tagged proteins were binding to the affinity beads, a Western blot was performed with antibodies against Mre11 and Rad50, respectively. The Rad50 antibody did not reveal any bands (data not shown). The Mre11 antibody showed that very low levels of recombinant Mre11 was expressed (Figure 35), but did neither bind to the His beads nor to the Strep beads. Instead, all the protein remained in the supernatant fraction indicating that the protein was not capable to bind to the beads. At this point the project had to be abandoned due to time constraints and due to the fact that the host group of Dr. Manuel Stucki had left the IVBMB.

6. Discussion

6.1. Purification of Mre11/Rad50-dimers using the Baculovirus expression system and tandem affinity purification

The goal of this thesis was to purify MR dimers for single-particle EM and biochemical analysis. Previously, the lab expressed and purified Mre11/Rad50 proteins that were both tagged with His. As result a variability of products were obtained, Mre11 and Rad50 monomers, MR dimers and clumps (most likely aggregates of partially insoluble or not correctly folded protein) of different composition. The reason for this outcome most likely lies in the fact that both Mre11 and Rad50 were equally affinity-tagged, which means that they would also bind to the same affinity matrix. Therefore, it was not possible to separate pure MR dimers from the monomers and probably also not from the aggregates of different composition. Thus, the idea of this project was to exchange the His tag on Mre11 to a Strep tag, which would allow tandem purification over different columns and thus, isolation of pure MR dimers. A Strep-tagged Mre11 expression bacmid was already available in the lab so we started directly with the baculovirus production. The first difficulty that came up here was that the SF21 cells were quite sensitive and tended to die fast, if the culture conditions were changed slightly. With more experience it was possible to increase the time in which the cells were in a condition that allowed performing experiments with them. The virus production went well and expression tests using the recombinant Mre11-Strep viruses revealed detectable protein expression on a Western Blot. But since the titer of the Mre11-Strep virus stock was rather low, we decided to amplify the Mre11 virus first. With the amplified viruses an initial co-infection was done. The cells were harvested and extracted with NP-40 to extract the proteins from the cells. The extract was then run over nickel beads and then over Streptavidin beads. From each step aliquots were taken and loaded on a SDS-Gel. This analysis showed that the first purification worked well, even with little protein. But the second step did not work at all because all the recombinant protein was found in the flow through fraction, which indicated that the proteins did not bind to the beads efficiently. Because the protein concentration was really low from the beginning we assumed that this might have caused the binding problem.

Therefore, it was decided to infect a larger number of cells and to do the purification with an ÄktaFPLC system. The first purification step on the ÄktaFPLC worked quite well, only the pressure indicator did not show any data, but since the extract wasn't too viscous, we assumed that the pressure wouldn't be too high. The other data curves (UV absorbance etc.) looked mostly as expected, only the protein peak in the elution fraction wasn't as high as anticipated.

The eluate was then loaded on a SDS-PAGE for analysis. The results showed that different proteins bound to the beads and that at the molecular weight position where Rad50 and Mre11 are expected to run, a faint band was visible. Western Blot analysis also showed that both Mre11 and Rad50 proteins were expressed and bound to the His beads. The pooled protein fractions from the first purification step were incubated with Strep beads after dialysis and bound proteins were eluted with SDS-Page loading buffer. Analysis of this second purification step revealed that the recombinant Mre11 and Rad50 proteins were only detectable in the lysate and flow through, respectively. Western blot analysis confirmed these results. Since we also had Streptavidin-coated Dynabeads available, we tried to purify the MR dimers with them. Dynabeads are thought to be more inert and to bind more protein than Strep agarose. But we essentially got the same results with them.

One possibility was that the protein concentration was too low in the eluate of the first purification step. Another possibility we considered was that the Strep tag sequence was either wrong or for some reasons not accessible (maybe due to sterical hindrance).

Therefore, the purification with a larger amount of extract was repeated and the Mre11-Strep transfer vector was sent for sequencing. The second purification showed very similar results as the first one. Moreover, the sequencing results revealed that the Strep-tag on Mre11 was indeed faulty. It comprised only a few amino acids, followed by a premature stop codon. This construct would lead to the expression of full length Mre11 without an affinity tag and thus explains our result that no recombinant protein was binding to the Strep beads.

It was then decided to start from scratch and generate a new Mre11 bacmid with the correct tag sequence at its C-terminus.

The insertion of the Mre11 cDNA into pFastBac1 together with the addition of a C-terminal Strep tag caused various difficulties. The biggest problem was that the Mre11 cDNA preferentially inserted into the vector in the wrong orientation (3’ – 5’). Different conditions were tried (vector insert ratios) but with no positive results. Therefore the strategy was changed and PCR was used to amplify the Mre11 cDNA and at the same time induce new restriction sites flanking the cDNA to insert it in the right position in front of the Strep tag. This eventually worked and two bacmid clones with the correct sequence could be isolated. Unfortunately though, we were not able to express Mre11 protein that bound to the Strep-affinityresin with this bacmid, even though it had the correct sequence. One possible reason for this is that the amounts of recombinant protein expressed by our recombinant baculoviruses were extremely low. It could barely be detected by Western blot and we didn’t detect the protein on Coomassie blue stained SDS gels. It is a known fact that low protein concentration can cause problems during purification. Unfor-

tunately, time didn't allow to optimize recombinant protein expression because the host group of Dr. Manuel Stucki moved to a different institute at the University Hospital Zürich.

In summary, even though the primary goal of this thesis could not be achieved due to technical difficulties and time constraints, it was nevertheless managed to correct the faulty Mer11-strep clone and to generate a Mre11-strep bacmid with the correct sequence. This new bacmid could now be used to optimize expression conditions to eventually isolate pure MR dimers for EM analysis.

7. Abbreviations

α	Anti
bp	Base pairs
BSA	Bovine serum albumine
CIP	Calf intestine phosphatase
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleosidetriphosphat
ds	Double stranded
DSB	DNA double-strand break
ECL	Enhanced chemiluminescence
E.coli	Escherichia Coli
EDTA	Ethylendimin-tetraacetic acid
FCS	Fetal calf serum
FPLC	Fast protein liquid chromatography
HEPES	4-(Hydroxyethyl) Piperazine-1-ethanesulonic acid
HR	Homologous recombination
HRP	Horseradish peroxidase
IPTG	Isopropyl-β-D-thiogalactopyranosid
LB-medium	Luria-Bertani-medium
MRE11	Meiotic recombination 11 protein
MRN-complex	Mre11-RAD50-NBS1-complex
NBS1	Nijmegen Breakage Syndrome 1 protein
NHEJ	Non-homologues end joining
NP40	Nonidet P40
OD	Optical Density

PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
RAD50	Radiation sensitive yeast 50
rpm	Rounds per minute
ss	Single stranded
SDS-PAGE	Sodium dodecylsulfate – polyacrylamid electrophotesis
SF21	Spodoptera frugiperda 21
TEMED	N,N,N',N'-Tetramethylethylendiamin
Tris	Tris-(hydroxymethyl)-aminomethan
UV	Ultraviolet
X-gal	bromo-chloro-indolyl-galactopyranoside

8. References

8.1. Literature

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8.2. List of figures

Figure 1: Czornak, K., Chughtai, S., Chranowska, K.H. (2008). Mystery of DNA repair: the role of the MRN complex and ATM kinase in DNA damage repair. J Appl Genet 49(4), pp. 383-396.

Figure 2: Kanaar, R., Wyman, C. (2008). DNA Repair by the MRN Complex: Break It to Make It. Cell 135, 14-16.

Figure 3: Rupnik, A., Lowndes, N.F., and Grenon, M. (2010). MRN and the race to the break. Chromosoma 199, 115-135.

Figure 4: Rupnik, A., Lowndes, N.F., and Grenon, M. (2010). MRN and the race to the break. Chromosoma 199, 115-135.

Figure 5: Rupnik, A., Lowndes, N.F., and Grenon, M. (2010). MRN and the race to the break. Chromosoma 199, 115-135.

Figure 6: Invitrogen Bac-To-Bac® Baculovirus Expression Systems Instruction Manual

Figure 20: One-STrEP^{TM}Kit: One-STrEP-tag Purification for the Isolation an Identification of Protein Complexes in Mammalian Cells, IBA BioTAGnology.

Figure 22: Invitrogen Bac-To-Bac® Baculovirus Expression Systems Instruction Manual

9. Appendix

9.1. Buffers, medias and solutions

Blue Dye	1ml Bromphenol blue 0.5 ml β-mercapto-ethanol (14.2M) add dH_2O to 10ml
Buffer 1	50mM Tris pH 7.0 0.5M NaCl 0.5% NP40 20mM Imidazole (10µl)
Buffer 2	50mMTris pH 7.0 0.5M NaCl 0.5% NP40 50-200mM Imidazol (100µl)
Buffer 3	50mM Tris pH 7.0 0.5M NaCl
Buffer A	249.375ml 1x PBS 0.5ml NaCl (5M) 0.125ml Triton 100
Buffer B	186.875ml 1x PBS 0.5ml NaCl (5M) 0.125ml Triton 100 62.5ml Imidazole (2M)
Buffer C	99.75ml 1x PBS 0.2ml NaCl (5M) 50µl Triton 100
Buffer D	99.55ml 1x PBS 0.4ml NaCl (5M) 50µl Triton 100
Destain	400ml MetOH 10ml Acetic Acid add dH_2O to 1000ml

Extraction buffer A	50mM Tris pH7.0 0.25M NaCl 0.5% NP40 10% Glycerol 1mM DTT
LB medium (Luria-Bertani medium)	10g Bactotryptone 5g Bacto-yeast extract 10g NaCl in 1l dH_2O adjust pH to 7.0 with NaOH
Lysis Buffer	99.25ml 1x PBS 0.2ml NaCl (5M) 50µl Triton 100 0.5ml Imidazole (2M)
6x Loading buffer	30ml Glycerol (87%) 0.5g Bromphenol blue 0.5g Xylen Cynol add dH_2O to 200ml
1kb Marker	30µl 1kb Marker 6x loading buffer add dH_2O to100µl
10x PBS (Phosphate-buffered saline)	80g NaCl 2g KCl 14.4g Na_2HPO 2.4g KH_2PO add dH_2O to 1l adjust pH to 7.4 with HCl
Ponceau S	2g Ponceau S 30g Trichloridacid 30g Sulfatsalicyl acid add dH_2O to 100ml

2x Running-gel buffer	150ml Tris (1M, pH 8.8) 2ml SDS (20%) add dH_2O to 200ml
10x Running buffer	60.6g Tris 288.2g Glycin add dH_2O to 2l
20% SDS	40g SDS add dH_2O to 200ml
2x SDS loading buffer	1ml Tris (1M, pH 6.8) 50µl β-mercapto ethanol 2ml SDS (20%) 200µl Bromphenol blue 2ml Glycerol (87%)
SOB	20g Bacto-Trypton 5g Bacto-yeast extract 0.5g NaCl add dH_2O to 1000ml 10ml KCl (250mM) adjust pH to 7.0 with NaOH 5ml $MgCl_2$ (5M) just before use
SOC	980ml SOB 10ml $MgSO_4$
Stacking-gel buffer	30.2ml Tris (1M, pH.8) 1.2ml SDS (20%) add dH_2O to 200ml
Stain	0.25g Coomassie Brilliant Blue R250 100ml MeOH 25ml Acetic acid add dH_2O to 250ml

1x TAE buffer	4.84g Tris 1.14g Gluacal acetic acid 2ml EDTA (0.5M, pH8) add H_2O to 1000ml
TB buffer	5ml Hepes (0.5M, pH 6.7) 3.75ml $CaCl_2$ (1M) 25ml KCl (2.5M) add dH_2O to 250ml adjust pH to 6.7 with KOH add 27.5ml $MnCl_2$ (0.5M) before use
1x TBS buffer	12.1g Tris 97.7g NaCl add H_2O to 1000ml
TBS-T	1l TBS 0.5ml Tween 20
Transfer buffer	100ml Running buffer (10%) 200ml Methanol add dH_2O to 1l

9.1. Primers and oligonucleotides

M13_for

5`-GTT TTC CCA GTC ACG AC -3`

M13_rev

5`-CAG GAA ACA GCT ATG AC -3`

Up_Mre11_BamH1

5’-CGG GAT CCA TGA GTA CTG CAG ATG CAC TTG ATG-3’

rev_pCR2.1_TOPO

5’-GTA AAA CGA CGG CCA GTG AAT TGT AAT ACG-3’

Up_Mre11_Kpn1

CGGGTACCATGAGTACTGCAGATGCACTTGATG

9.2. Sequencing results

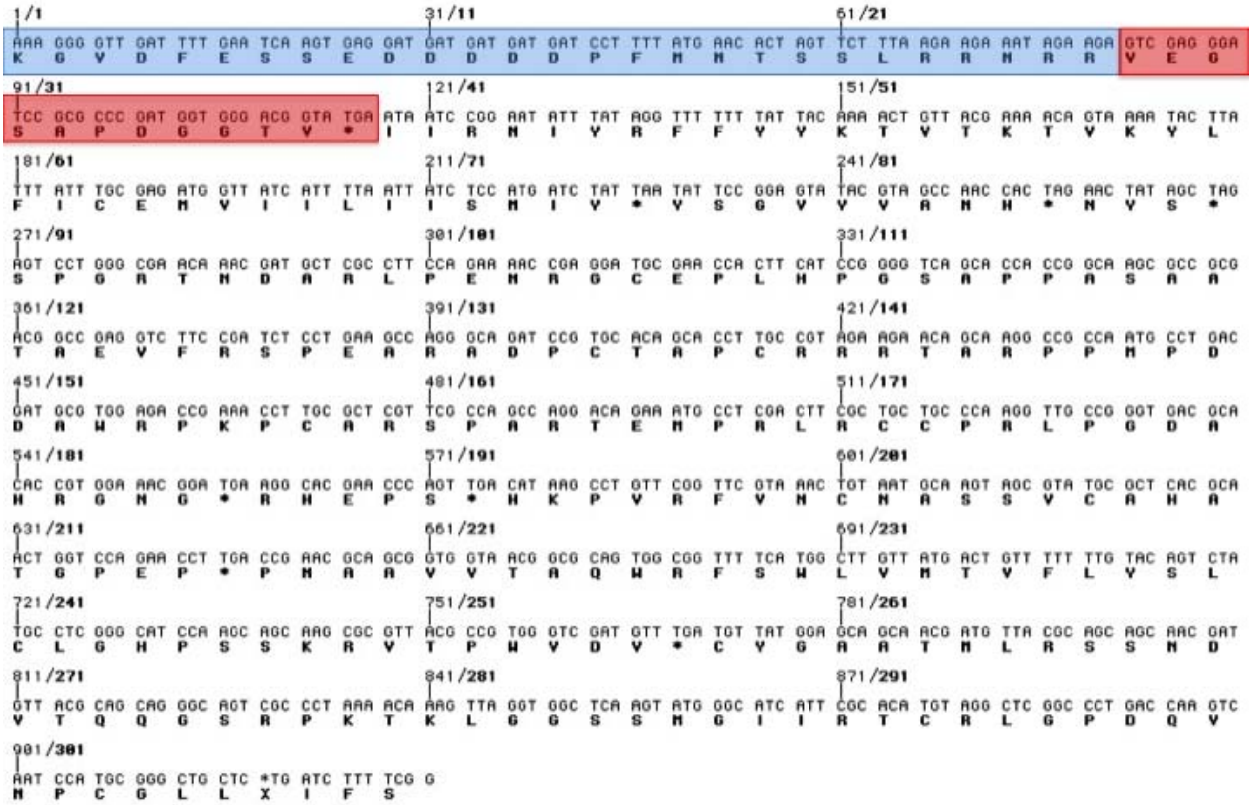

Figure 36: Sequencing result pFastBac-Nbs1-Mre11-Strep. Light blue highlighted Mre11 C-terminus. Light red highlighted truncated C-terminal tag.

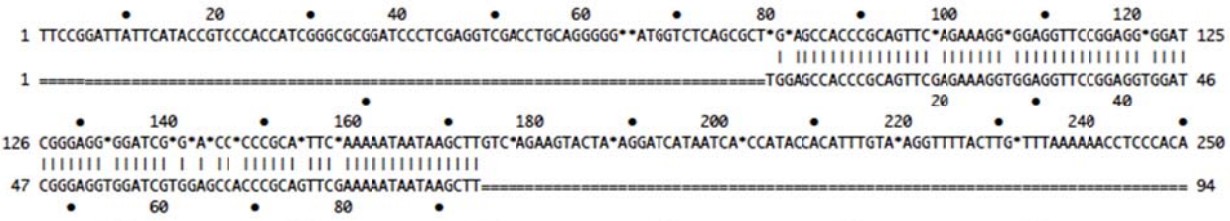

Figure 37: Sequencing result pFastBac-Strep aligned with Strep-sequence.

10. Curriculum vitae

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